Neutrophil dysfunction in Acute and Chronis Liver Failure

Taylor, Nicholas James

Awarding institution:
King's College London

Neutrophil Dysfunction in Acute and Chronic Liver Failure

By

Dr Nicholas James Taylor BSc MBBS

A THESIS
SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE OF THE UNIVERSITY OF LONDON

Institute of Liver Studies
Division of Transplantation Immunology and Mucosal Biology
King's College London School of Medicine
At King's College Hospital, Denmark Hill, London, SE5 9RS

October 2016

I, Nicholas Taylor, declare that the work presented in this thesis is my own**.**

Nicholas Taylor

The greatest challenge to any thinker is stating the problem in a way that will allow a solution
- Bertrand Russell

Abstract

Patients with liver disease are susceptible to sepsis which is associated with poor outcomes and high mortality. The immunopathology includes a defective innate immune response to invading microbes and an exaggerated systemic inflammatory response. Neutrophils, potential effectors of this differential response, undergo priming leading to alterations in phagocytic capacity and oxidative burst (OB). Ammonia-induced osmotic stress and a circulating pro-inflammatory milieu mediated through phosphorylation of the p38-MAPK signalling pathway have been postulated to be critical events in the development of neutrophil dysfunction in liver disease.

This longitudinal case-control study characterised neutrophil morphology, phenotype and function *ex-vivo* in patients with acute liver failure (ALF) and cirrhosis. The role of ammonia–induced osmotic stress in relation to the p38-MAPK pathway was explored.

25 patients with ALF and 67 patients with cirrhosis were compared with healthy (HC) and sepsis controls (SC). Utilising flow cytometry, neutrophil phenotype was determined by surface CD16 and CD11b expression, phagocytosis quantified with FITC-labelled *E.coli*, OB pre/post-exposure to *E.coli* by 123-rhodamine fluorescence, chemotaxis by the number of cells migrating towards a fMLP concentration gradient and cell volume determined by forward scatter characteristics with confirmatory transmission electron microscopy. Plasma cytokine concentrations were determined using ELISA. Finally, neutrophil function assays were repeated after pre-incubation with p38-MAPK modulators and intracellular levels of total and phosphorylated p38-MAPK were determined.

My data demonstrate that circulating neutrophils in ALF have impaired bactericidal function similar to that seen in severe sepsis. Neutrophil function indices are important biomarkers in ALF and may be implicated in the development of organ dysfunction and increased susceptibility to sepsis. Likewise, neutrophils in patients with cirrhosis are dysfunctional and predict the development of infection, organ dysfunction and survival at 90-days and 1-year. Neutrophil swelling correlates with peak arterial concentration and can be abrogated *ex-vivo* following incubation with the p38-MAPK agonist isoproterenol.

Acknowledgements

I would like to acknowledge the help and support of the following:

Dr Debbie Shawcross, my first supervisor, for her constant support and boundless enthusiasm for her science and for many hours she spent in the laboratory showing me the ropes and perhaps more critiquing my writing. She has introduced me to a fascinating area of liver disease and cultured my curiosity about a miraculous cell often taken for granted. Without her I simply would not have had the perseverance to complete the thesis and for that I am forever grateful.

Dr Yun Ma who has been an excellent foil as my second supervisor acting as an oracle of immunological knowledge and offering constructive support as well as casting her critical eye over my scientific methodology and flow-plots.

I would like to thank Godhev Manakkat Vijay for his assistance in performing the neutrophil assays and general laboratory support as well as the kind donation of images from his own thesis. I seem to have infected him with the same enthusiasm for neutrophils, liver disease and flow-cytometry, which he has now taken to the next level for that I can only apologise.

I would like to thank Anirudh Nishtala for his assistance in performing the neutrophil assays and transmission electron micrographs, Dr Mark McPhail for his advice regarding the statistical analyses and Dr Munther Hussain for his help and technical guidance in performing ELISA. Thanks also goes to Dr Daniel Abeles for his technical advice and scientific banter which has challenged my views and offered new perspectives on the immunopathology in liver disease as well as his help in experimental technique and patient recruitment. Dr Abeles however will have to concede that there is more to the immune system than monocytes and PBMC's.

Dr Zudin Zudan for his help in the recruitment of septic control patients from the general ICU. Professor Alice Warley and the staff at the centre for Ultrasructural imaging at Guy's hospital for helping me to produce the transmission electron micrographs.

I would like to offer special thanks to Professor Julia Wendon, Dr Georg Auzinger and Dr William Bernal for all they have taught me about liver disease and the management of very sick patients in the Liver Intensive Care Unit and for creating a

very special unique place where doctors can learn and gain experience from exceptional role models.

The help and support of all the staff on the Liver Intensive Care Unit, liver outpatients, Todd and RD Lawrence ward has been immense and invaluable.

A Young Investigator Grant awarded to Dr D.L Shawcross and Professor J.A Wendon from The Intensive Care Society in 2008 partly funded this study. Additional laboratory consumables were also funded from The Institute of Liver Studies Liver Intensive Care Charitable Fund.

I would like to acknowledge Medical Research Council (MRC) Centre for Transplantation, King's College London, UK – MRC grant no. MR/J006742/1 and for support from the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London.

I dedicate this thesis to my wife Gisela and family, Jack, Tobias and little Orlaith who have had to suffer my obsession with neutrophils and the trials and tribulations that laboratory work brings as well as the many hours spent writing.

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Abbreviations

Acetaminophen acute liver failure (AALF)

Acute alcoholic hepatitis (AAH)

Acute kidney injury (AKI)

Acute liver failure (ALF)

Acute lung injury (ALI)

Acute-on-chronic liver failure (AoCLF)

Acute Physiology and Chronic Health Evaluation II (APACHE II)

Adult respiratory distress syndrome (ARDS)

Alanine aminotransferase (ALT)

Alcoholic hepatitis (AH)

Alcohol-related liver disease (ARLD)

Allophycocyanin-Cyanin 7 (APC-Cy7)

Anti-diuretic hormone (ADH)

Area under the receiver operated curve (AUROC)

Aspartate aminotransferase (AST)

Autoimmune hepatitis (AIH)

Bacterial permeability-increasing protein (BPI)

B-cell lymphoma extra-large (Bcl-X)

Becton Dickinson (BD)

Bone marrow (BM)

Bovine serum albumin (BSA)

Bronchoalveolar lavage (BAL)

CC-chemokine ligand (CCL)

CC-chemokine receptor (CCR)

California (CA)

Central nervous system (CNS)

Child-Pugh class A (CPA)

Child-Pugh class B (CPB)

Child-Pugh class C (CPC)

Child-Pugh score (CPS)

Circulatory assist device (CASD)

Cirrhosis associated immune dysfunction syndrome (CAIDS).

Chronic granulomatous disease (CGD)

Chronic liver disease (CLD)

Cluster of differentiation (CD) – CD11b, CD16, CD62L

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Complement component-5a (C5a)

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Cleavage fragment C3b [iC3b])

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Fluorescence-activated cell sorting Canto II analyser (FACSCantoII)

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Glasgow Coma Scale (GCS)

Glutathione (GSH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glycoprotein 91 phagocyte oxidase ($gp91^{phox}$)

Gram-negative cocci (GNC)

Gram-positive cocci (GPC)

Granulocyte colony stimulating factor (G-CSF)

Granulocyte-macrophage colony stimulating factor (GM-CSF)

Guanosine-5'-triphosphate (GTP)

Healthy control (HC)

Hepatic encephalopathy (HE)

Hepatorenal syndrome (HRS)

Hepatic venous pressure gradient (HVPG)

Hepatitis A virus (HAV)

Hepatitis B virus (HBV)

Hepatitis C virus (HCV)

Hepatitis E virus (HEV)

High-density lipoprotein (HDL)

High mobility group box-1 (HMGB-1)

Horseradish-peroxidase (HRP)

Human leukocyte antigen-DR (HLA-DR)

Immunoglobulin (Ig)

Immunoglobulin-A (IgA)

Immunoglobulin-G (IgG)

Inositol 1,3,4-triphosphate (IP3)

Intensive care unit (ICU)

Interferon (IFN) (interferon-gamma [IFNγ])

Interleukin (IL) – [IL-6, IL-8, IL-10, IL-17]

Interleukin-8 (IL-8, also known as CXCL8)

Interleukin-1 receptor (IL-1R)

Interlukin-8 receptor (IL-8R)

Interleukin-1 receptor-associated kinase (IRAK)

International normalised ratio (INR)

Interquartile range (IQR)

Intracellular-adhesion molecule-1 (ICAM-1)

Intracranial pressure (ICP)

c-jun N-terminal kinase (JNK)

Keratinocyte derived chemokine (KC)

Left lower (LL)

Leukocyte adhesion deficiency-type1 (LAD1)

Leukocyte adhesion deficiency-type 2 (LAD2)

Leucocyte function antigen-1 (LFA-1)

Leucocyte rich plasma (LRP)

Leukotriene B4 (LT-B4)

Leukotriene B4 receptor (BLT1)

Lipoteichoic acid (LTA)

Lipopolysaccharide (LPS)

Lipopolysaccharide binding protein (LBP)

Liver sinusoidal endothelial cells (LSEC)

Liver transplantation (LT)

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Macrophage inflammatory protein-2 (MIP-2)

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Mean fluorescence intensity (MFI)

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Mitogen activated protein kinase kinase (MKK)

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Neutrophil phagocytic capacity (NPC)

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Oxidative burst (OB)

Partial atmosphere (Pa)

Pathogen-associated-molecular patterns (PAMP)

Pattern recognition receptors (PRR)

Peripheral blood mononuclear cells (PBMC)

Phagocyte oxidase protein 22 ($p22^{phox}$)

Phagocyte oxidase protein 47 ($P47^{Phox}$)

Platelet activating factor (PAF)

Platelet activating factor receptor (PAFR)

Phagocyte oxidase (phox)

Phagocytosis-induced cell death (PICD)

Phosphate buffered saline (PBS)

Phosphoinositide-3 kinase (PI-3K)

Phospholipase-A2 (PLA_2)

Phospholipase C (PLC)

Photomultiplier tubes (PMT)

Phycoerytherin (PE)

Phorbol 1,2-myristate 12-acetate (PMA)

Polymorphonuclear (PMN)

Primary biliary cirrhosis (PBC)

Primary sclerosing cholangitis (PSC)

Propidium iodide (PI)

Protein kinase A (PKA)

Protein kinase C (PKC)

P-selectin glycoprotein ligand (PSGL-1)

p38-Mitogen activated protein kinase (p38-MAPK)

Radioimmunoprecipitation assay (RIPA)

Ras-related C3 botulinum toxin substrate 1 (Rac-1)

Ras-related C3 botulinum toxin substrate 2 (Rac-2)

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Receiver operated curve (ROC)

Renal replacement therapy (RRT)

Renin-angiotensin aldosterone system (RAAS)

Reticulo-endothelial (RE)

Revolutions per minute (RPM)

Right lower (RL)

Rough endoplasmic reticulum (RER)

Septic controls (SC)

Sequential Organ Failure Assessment (SOFA)

Side scatter (SSC)

Sodium dodecyl sulphate (SDS)

Soluble N-ethylmaleimide-sensitive-fusion protein attachment protein receptors (SNARE)

Soluble tumour necrosis factor receptor (sTNFR)

Spleen tyrosine kinase (Syc)

Spontaneous bacterial peritonitis (SBP)

Spontaneous oxidative burst (SOB)

Stromal-cell-derived factor (SDF-1)

Systemic inflammatory response syndrome (SIRS)

Subacute liver failure (SALF)

Superoxide dismutase (SOD)

Transmission Electron Microscopy (TEM)

Toll-like receptors (TLR) – [TLR-2, 3,4,9]

Transforming growth factor-β (TGF-β)

Triggering receptor on myeloid cells (TREM-1)

Tumour necrosis factor α (TNFα)

Tumour necrosis factor receptor (TNFR)

Tumour necrosis factor receptor-associated factor 6 (TRAF-6)

T-regulatory cells (T-regs)

United Kingdom (UK)

United Kingdom End-Stage Liver Disease score (UKELD)

Vascular cell adhesion protein-1 (VCAM-1)

Very-late antigen-4 (VLA-4)

White cell count (WCC)

Chapter 1 - Neutrophil structure, function, morphology and life-cycle

1.1 Introduction

Neutrophils are highly motile phagocytic effector cells of the innate immune system. They were first discovered by Elie Metchnikoff in 1884 when he inserted rose thorns into starfish larvae and observed wandering cells accumulate at the puncture site and engulf particles; a process subsequently termed phagocytosis. (Metchnikov (1884) The larger cells were named macrophagocytes (macrophages) with the smaller cells microphagocytes; that name subsequently changing to granulocytes of which neutrophils are the most abundant. Neutrophils constitute the first line of defence against bacterial and fungal infection, and play a critical role in the response to cellular damage (Witko-Sarsat et al., 2000). The speed of the neutrophil response and varied arsenal of anti-microbial capabilities allow them to effectively eradicate the majority of infections encountered. (Segal, 2005) The importance of the neutrophil response to infection is highlighted in patients with functional neutrophil deficiency syndromes such as chronic granulomatous disease, leucocyte adhesion deficiency and chemotherapy-induced neutropenia; these conditions can result in fulminant infection from bacterial and fungal pathogens, frequently from opportunistic organisms (Schelenz et al., 2012). Recently there is evidence that a number of inflammatory conditions, such as sepsis and liver failure, are associated with a quantitative and qualitative antimicrobial dysfunction of neutrophils. (Brown et al., 2006, Rajkovic and Williams, 1986) Furthermore neutrophil antimicrobial dysfunction can simultaneously be associated with an over exuberant neutrophilic response leading to cellular damage and organ dysfunction. (Annane et al., 2005)

1.2 Neutrophil function

Functionally circulating neutrophils exist in a 'resting state'. Neutrophils can undergo rapid activation and recruitment at sites of tissue inflammation due to infection, trauma or an autoimmune reaction. Neutrophils migrate to sites of inflammation through a process called 'chemotaxis' being attracted by soluble factors, such as Interleukin-8 (IL-8) [also known as *neutrophil chemotactic factor*], Interferon (IFN) γ, complement proteins, and molecules released from damaged tissues and macrophages. (Williams et al., 2011) Once at the site of inflammation neutrophils come into close proximity to the vascular endothelium (margination) allowing then to

receive further chemokine signalling facilitating firm endothelial adhesion, subsequent activation and migration across the endothelium (diapedesis). (Guthrie et al., 1984, Follin et al., 1991) Upon entering the tissues neutrophils undertake three critical antimicrobial functions resulting in removal of invading pathogens and cell debris. Firstly neutrophils are able to engulf microbes and particles in a process termed phagocytosis. (Lee et al., 2003) Following ingestion neutrophils proceed to eliminate ingested material by generating large quantities of reactive oxygen species (ROS) through activation of nicotinamide adenine dinucleotide phosphatase-oxidase (NADPH-oxidase), a process termed respiratory or oxidative burst (Dahlgren and Karlsson, 1999, Baldridge, 1933). Secondly neutrophils can directly release anti-microbial molecules such as defensins and serine proteases after fusion of granules with the plasma membrane. Recently a third and slightly curious antimicrobial function of neutrophils has been discovered; they appear to release an extracellular web-like structure composed of DNA and serine proteases in response to lipopolysaccharide (LPS) and IL-8. These neutrophil extra-cellular traps (NETs) snare and kill microbes whilst minimising damage to surrounding cells. (Brinkmann et al., 2004, Wartha et al., 2007) Having undergone respiratory burst neutrophils become apoptotic and their lysed remnants constitute pus.

The release of ROS and serine proteases into the extracellular space from activated neutrophils can generate 'collateral damage' to surrounding cells. This bystander damage can contribute significantly to tissue destruction in both acute and chronic disease processes, such as acute lung injury (ALI), emphysema and rheumatoid arthritis (Holman and Saba, 1988). Interestingly, the absence of alpha1-antitrypsin, the natural inhibitor to elastase, one of the serine proteases, can lead to excessive tissue damage resulting in emphysema and hepatic disease. There is therefore a trade-off between ensuring an adequate response to eradicate invading microbes whilst avoiding excessive damage to bystander cells through an overly aggressive response. Indeed, microbes may manipulate neutrophils to shift this balance.

1.3 Neutrophil morphology

Neutrophils or polymorphonuclear leukocytes are granulocytes of the myeloid lineage and are the most common form of leukocyte in humans and other primates. On a blood smear neutrophils are 12-15 microns in diameter with poorly staining cytoplasmic granules and lysosomes (variable or "heterophil" staining) differentiating them from the other granulocytes, eosinophils and basophils. Neutrophils also possess a characteristic variably segmented or lobar nucleus. Immature forms have one (band forms) or two lobes but mature forms may have 3 to 5 lobes, connected together with fine threads of nuclear material. An excessive number of immature forms on a blood film is termed 'left shift' and is indicative of infection. During infection neutrophils also exhibit toxic granulation with densely stained granules within the cytoplasm, vacuolisation of the cytoplasm and Dhole bodies (residual ribosomes) [Figure 1-1].

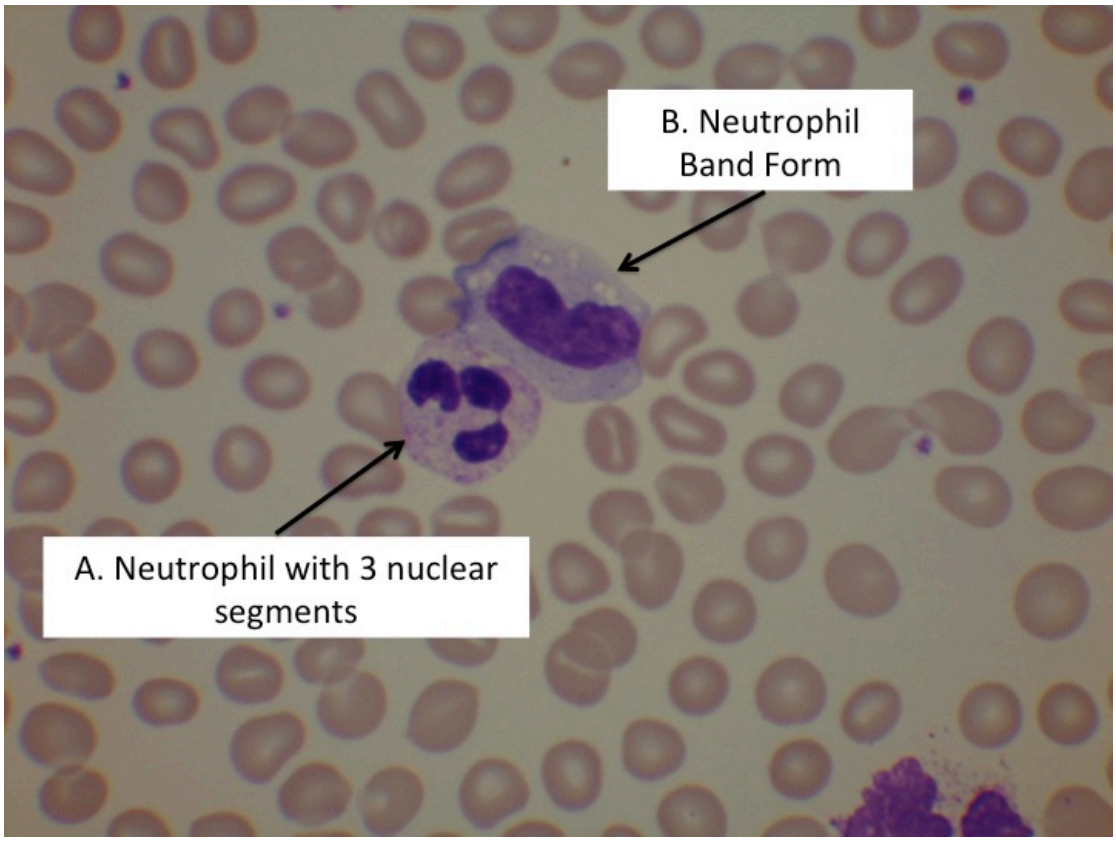


FIGURE 1-1 LIGHT MICROSCOPY IMAGES OF A NORMAL NEUTROPHIL (A) WITH 3 NUCLEAR SEGEMENTS AND AN IMMATURE OR BAND FORM (B) FROM A HEALTHY CONTROL.

Courtesy of Godhev Manakkat Vijay, and Professor Barbara Bain (Manakkat Vijay et al., 2015)

1.4 Neutrophil life cycle and trafficking from bone marrow to peripheral blood

Neutrophils constitute 50-70% of circulating leucocytes with the standard range for peripheral blood cell counts being 2.5-7.5 x 10^9/L. In fact circulating neutrophils represent less than 2% of total body neutrophils, the majority are retained within the bone marrow (BM) with release into the circulating pool being tightly controlled. Neutrophils are produced in vast quantities upto 5-10x10^{10} per day (Summers et al., 2010) and undergo development over 14-days in the BM. Upon release into the circulation the average neutrophil life-span is only 5.4 days (Pillay et al., 2010). Thereafter in the absence of bacterial infection, ageing neutrophils shrink into apoptotic bodies, undergoing phagocytosis by macrophages in reticuloendothelial organs or the BM. (Savill et al., 1989) Under stressful conditions circulating neutrophil numbers can rise significantly, so called 'stress granulopoiesis'. Neutrophil numbers can be increased through egress of mature neutrophils from the BM, reduced neutrophil apoptosis and subsequent clearance. The retention of neutrophils within the BM is regulated by the CXC chemokine receptors CXCR2 and CXCR4 expression on the neutrophil cell surface (Christopher and Link, 2007). WHIM disease is a condition where autosomal dominant mutations of CXCR4 result in a disease characterised by warts, hypogammaglobulinaemia, infections and myelokathexis (retention of mature neutrophils within the BM). This is due to a mutation in the CXCR4 gene generating truncated forms of the protein resulting in enhanced signalling. (Hernandez et al., 2003) CXCR4 activation retains neutrophils within the BM whereas CXCR2 facilitates egress. Interestingly as neutrophils mature they have reduced surface expression of CXCR4 and the β1-Integrin, very-late antigen-4 (VLA4), ensuring the preferential release of these neutrophils from the BM. The dominant production of the CXCR4 ligand stromal-cell-derived factor (SDF-1) from osteoclasts results in neutrophil retention. Granulocyte colony stimulating factor (G-CSF) mobilises neutrophils from the BM during sepsis by inhibiting osteoclast production of Stromal cell-derived factor-1 (SDF-1) and increasing the production of CXCR2 ligands, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2). (Semerad et al., 2002) In addition, granulocyte-macrophage colony

stimulating factor (GM-CSF) and other circulating chemokines such as IL-6 and IL-8 appear to play critical roles in stress granulopoiesis (Zhan et al., 1998, Kopf et al., 1994, Starckx et al., 2002).

1.5 Surface receptor expression

Circulating neutrophils are critically dependent on their surface receptors to rouse them from their normal 'resting state' with associated low metabolic activity. Firstly to a 'primed' state of enhanced surveillance and finally to a fully 'activated' state associated with a massive increase in metabolic functioning. Neutrophils are maintained in a resting state in order to prevent uncontrolled release of ROS, proteases, and collateral tissue damage. Whilst in this 'resting state' they remain constantly vigilant, through the expression of an array of pattern recognition receptors (PRR), for the presence of molecular signatures indicating cellular damage or infection. In order to activate neutrophils and the NADPH-oxidase, the principle enzyme involved in respiratory burst, neutrophil surface receptors need to bind specific ligands. Firstly circulating neutrophils can be 'primed' by low 'non-activating' concentrations of agonists such as GM-CSF, G-CSF, tumour necrosis factor α (TNFα), IL-6 and IL-8 and results in a variety of biochemical changes. (Condliffe et al., 1998) Priming results in a greatly enhanced neutrophil response to subsequent circulating stimuli or opsonised particulate matter which leads to a functional response 'activation'. Once 'activated' neutrophils vastly increase their metabolic activity, undergoing a so-called 'respiratory burst', thus facilitating them to perform defined functions including endothelial transmigration, phagocytosis, activation of the NADPH-oxidase, degranulation, generation of lipid mediators (e.g. PAF and LTB4) and cytokines. (Vignais, 2002) This two-stage activation process again ensures that these highly toxic cells are only fully operational at sites of inflammation minimising collateral tissue damage.

Neutrophils are 'primed' and 'activated' through the binding of ligands to PRRs expressed on the neutrophil surface; these include Toll-like receptors (TLR), C-type lectin receptors, N-Formyl-methionyl-leucyl-phenylanine (fMLP) receptors and scavenger receptors. (Gordon, 2002) PRRs recognise highly conserved molecular signatures which indicate infection through pathogen-associated-molecular patterns (PAMP), and cellular damage through disease associated molecular patterns (DAMP)

[for ligand-receptor interactions see Table 1-1]. For particulate matter immunoglobulin (Ig) fragment crystallisable (Fc)-receptors (Cluster of Differentiation [CD] 16, CD32, CD64) and complement receptors (CD31 and CD88) will bind to circulating immunoglobulin and activated complement molecules bound to pathogens. The complement protein C5a is an important priming molecule. Neutrophils have a plethora of diverse chemoattractant receptors that overlap in a seemingly redundant fashion. These include chemokine receptors (CXCR, CCR), eicosanoid receptors and cytokines receptors (e.g. IL-1R) allowing 'priming' and 'homing' of neutrophils to sites of infection and inflammation. Stimulation of G-protein coupled chemotactic receptors trigger fusion of secretory vesicles with the plasma membrane and up-regulate signal transduction pathways, resulting in augmentation of neutrophil adhesion and the production of reactive oxygen intermediates [Figure 1-2]. (Jaeschke, 2006, Takeda et al., 2003)

Table 1-1 Neutrophil cell surface receptors and ligands

Receptor Group	Neutrophil cell surface receptors	Ligand
Pattern recognition receptors (PPRs)	Toll-like receptor-2 (CD282) Toll-like receptor-4 (CD284) Toll-like receptor-5 (CD285)	Lipoteichoic acid and others Lipopolysaccharide (LPS) Flagellin
	CD14 (in complex with TLR-4)	LPS
	C-type lectins Mannose binding receptor	 Mannose binding lectin (MBL)
	Scavenger receptor	CD36
	Triggering receptor expressed on myeloid cells (TREM-1)	Unknown
	N-Formyl peptide receptor (FPR)	fMLP
Immunoglobulin-Fcγ receptor (FcγR)	CD16 – low affinity CD32 – low affinity CD64 – high affinity	Immunoglobulin-G Fc region Immunoglobulin-G Fc region Immunoglobulin-G Fc region
Chemokine receptors (CXCR)	CXCR1 CXCR2 CXCR4	IL-8 (CXCL8) and CXCL6 CXCL1-7 SDF-1α
Complement receptors	CD88 Complement receptor CD31/CR1	C5a C3b
Eicosanoid receptors	BLT1 (Leukotriene B4 receptor) Platelet activating factor receptor (PAFR)	Leukotriene B4 Platelet activating factor (PAF)
Selectins	CD62-L (L-selectin)	Oligosaccharide
	P-selectin glycoprotein ligand-1 (PSGL-1)	P-selectin
β1-Integrins	CD49d/CD29 (VLA-4) – immature forms and sepsis	CD106 (VCAM-1) Fibronectin
β2-Integrins	CD11a/CD18 (leucocyte functional antigen-1) [LFA-1]	CD54 (ICAM-1)
	CD11b /CD18 (macrophage-1) [Mac-1]	CD54 (ICAM-1) iC3b

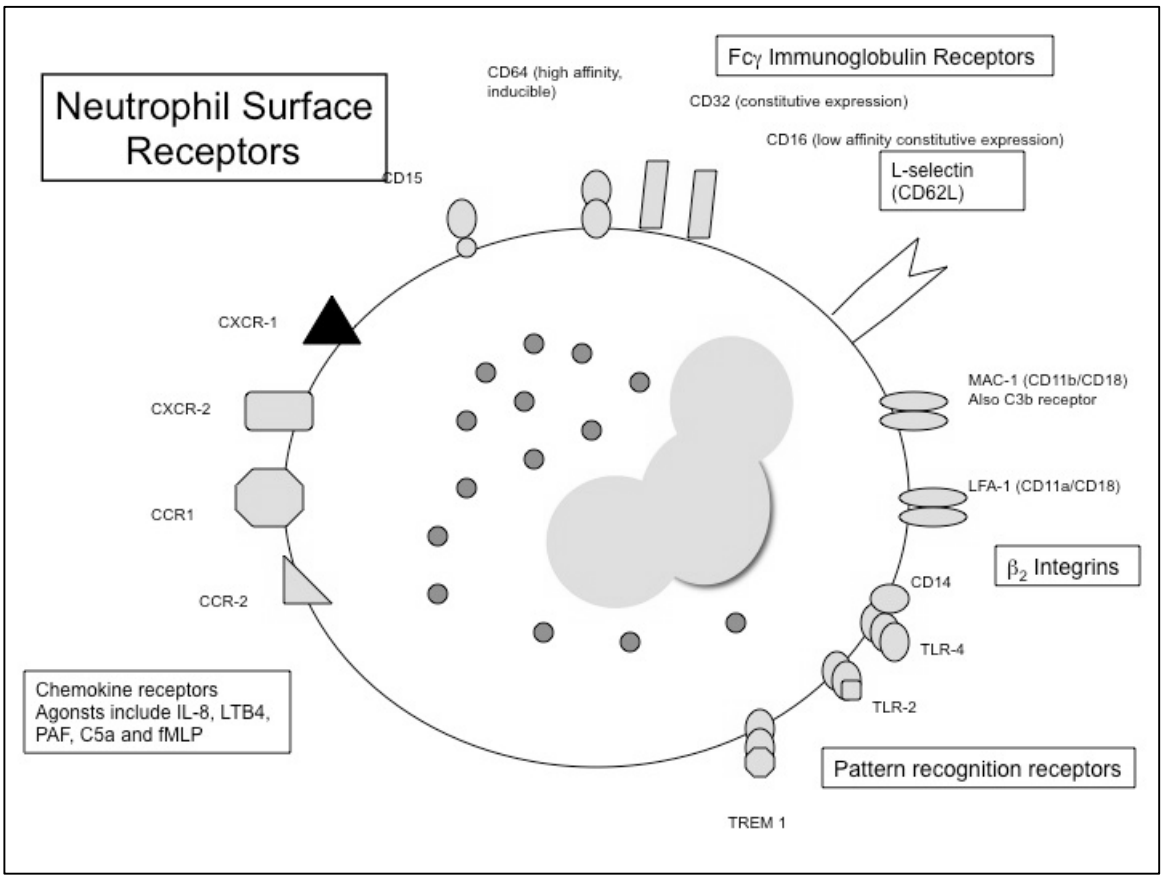


FIGURE 1-2 NEUTROPHIL CELL SURFACE RECEPTORS

Abbreviations: Fcγ - fragment crystallisable gamma; CD - cluster of differentiation; CXCR - CXC chemokine receptor; CCR - CC chemokine receptor; MAC-1 – Macrophage 1; TLR – Toll-like receptor; LFA-1 – Leukocyte function antigen 1; TREM 1 – triggering receptor expressed on myeloid cells 1; IL-8 – interleukin-8; LT-B4 – leukotriene B4; PAF-platelet activating factor; C5a – complement component 5a; fMLP – N-Formyl-methionyl-leucyl-phenylalanine.

1.6 Toll-like receptors and down-stream second messenger systems

TLRs are the most studied and perhaps best understood of the PRRs and highlight some of the critical steps involved in neutrophil activation. TLRs are highly conserved in evolutionary terms and regulate the major pro-inflammatory and host defence functions of human neutrophils as well as other immune cells such as macrophages and T-cells. They are membrane signalling receptors and key-signal transduction molecules in neutrophils regulating functions such as migration, activation and apoptosis in response to PAMPs such as LPS, lipoteichoic acid (LTA) and peptidoglycans (Ulevitch, 1999). Interestingly, TLR2 but not TLR4, expression is increased on the surface of neutrophils by pro-inflammatory cytokines (Kurt-Jones et al., 2002). TLR4 activation is complex and requires the binding of LPS in a multi-step process. LPS first associates with soluble LPS-binding protein (LBP), which facilitates complex formation with CD14. Once LPS binds to CD14, LBP dissociates, and the LPS-CD14 complex physically associates with TLR4. After ligand binding, a complex downstream pathway is activated that can be myeloid differentiation factor-88 (MyD88) dependent or independent. In the MyD88-dependent pathway, interleukin-1 receptor-associated kinase (IRAK), and tumour necrosis factor receptor-associated factor 6 (TRAF-6) are sequentially activated. These adaptors mediate the activation of a number of intracellular kinases including p38-mitogen activated protein kinase (p38-MAPK), extracellular signalling-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and phosphoinositide-3 kinase (PI3K). (Takeuchi and Akira, 2001) Ultimately nuclear transcription factor-κB (NF-κB) is activated which translocates to the nucleus and up-regulates the expression of a number of pro-inflammatory cytokines.

Activation of TLRs by PAMPs facilitates neutrophil recruitment by upregulation of endothelial adhesion molecule expression on endothelium directly or indirectly, via cytokine release from other tissues such as macrophages. TLR activation leads to down regulation of IL-8R, CXCR1 and CXCR2 facilitating recruitment and localisation of these cells to sites of infection and inflammation (Mullarkey et al., 2003, Meng et al., 2004). LPS activation of TLR4 is a potent activator of neutrophils, causing p38-MAPK activation, L-selectin shedding, CD11b expression, respiratory burst and the generation of cytokines e.g. IL-8 (Sabroe et al., 2003). TLR2 and TLR4 activation has been shown in a large number of studies to

delay Fas receptor ligation and constitutive apoptosis. LPS priming *in vivo* can delay apoptosis in models of bacterial sepsis. Interestingly, the increased neutrophil apoptosis induced by the influenza-A virus in the presence of *Streptococcus pneumonia* is thought to be the underlying mechanism whereby patients develop secondary pneumonia during viral infection, a frequent cause of death in infected patients. (Hajishengallis, 2011) LPS stimulation appears to have a modest direct effect in delaying neutrophil apoptosis; however indirect effects on other cells such as monocytes can produce a synergistic effect. (Walmsley et al., 2004) Direct effects are mediated through NF-κB, p38-MAPK and ERK (Sabroe et al., 2003).

Signalling cross talk between innate immune receptors such as TLR2 and C-type lectin receptors in the immune response against fungal pathogens allows amplification of weak PAMP signals. Additionally, cross talk can lead to suppression of TLR-induced pro-inflammatory responses classically following glucocorticoid and adenosine receptor signalling [Figure 1-3]. (Hajishengallis and Lambris, 2011)

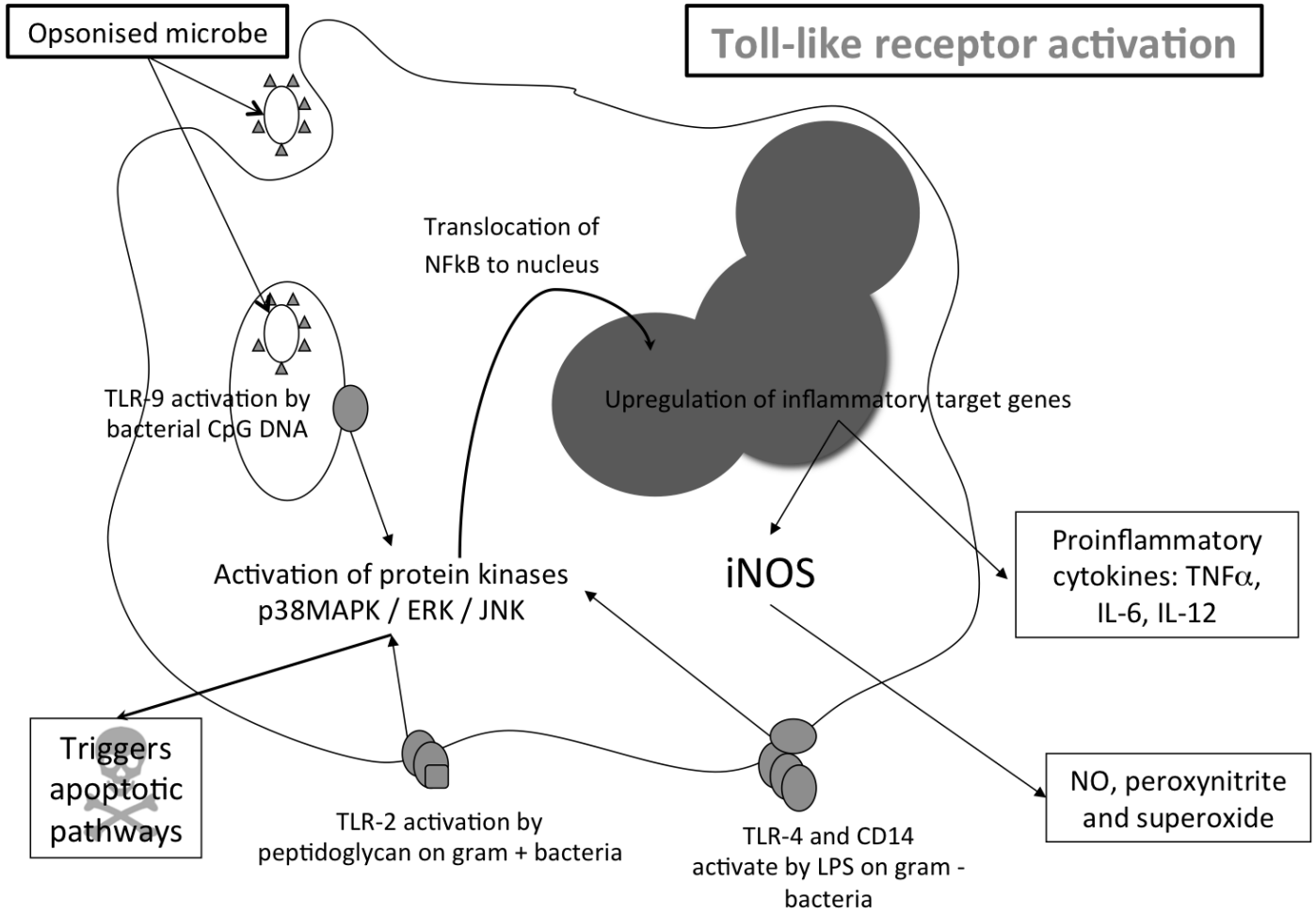


FIGURE 1-3 EFFECTS OF TOLL-LIKE RECEPTOR LIGATION IN NEUTROPHILS.

1.7 p38-MAPK neutrophil activation

MAP kinases play an important role as downstream effectors of neutrophil activation, mediating intracellular signal transduction and regulating cellular function in response to a variety of stimuli. Studies have demonstrated that p38-MAPK is involved in an intracellular kinase cascade that regulates stress-activated signal transduction. (Zarubin and Han, 2005) Surface receptor ligation appears to be the principle activation step in the p38-MAPK cascade: receptors include PRRs, such as TLRs and fMLP, pro-inflammatory cytokine receptors such as tumour necrosis factor receptors and Interleukin-1 receptor (IL-1R), and selectin binding to P-selectin glycoprotein ligand (PSGL-1) [Figure 1-4]. (Doyle et al., 2004, Zu et al., 1998, Hidari et al., 1997) Signal transduction occurs through a kinase cascade resulting in the rapid activation of the downstream kinases MAP kinase kinase (MKK) 3 and MKK6 and subsequently p38-MAPK. (Kumar et al., 2003) p38-MAPK subsequently stimulates Mitogen activated protein kinase activated protein (MAPKAP) kinase 2 and/or MAPKAP kinase 3. (Ono and Han, 2000) These kinases in turn induce the phosphorylation of small heat-shock proteins. In addition p38-MAPK can phosphorylate specific transcription factors *in vitro* and in intact cells. p38-MAPK signalling has been shown to involve the post-transcriptional regulation of cytokines such as TNFα and also cycloxygenase-2. (Dean et al., 1999) TNFα appears to specifically activate p38-MAPK; other general activators of kinase systems include GM-CSF, fMLP, Phorbol myristate acetate (PMA) and inomycin. The specific inhibitor of p38-MAPK SB203580 inhibits neutrophil p38-MAPK stimulation by TNFα including IL-8 production and superoxide generation (Zu et al., 1998). Selective inhibition of p38-MAPK reduced neutrophil chemotaxis and superoxide production in response to fMLP. Interestingly corticosteroids have been shown to inhibit p38-MAPK through expression of MAPK phosphatase-1, expression of which is also induced by IL-1, acting as a putative negative feedback loop. (Lasa et al., 2002) TLR-mediated induction of scavenger receptors (SRs) occurs through MyD88, IRAK4, and p38-MAPK. Activation of this pathway is essential for TLR promotion of phagocytosis (Doyle et al., 2004).

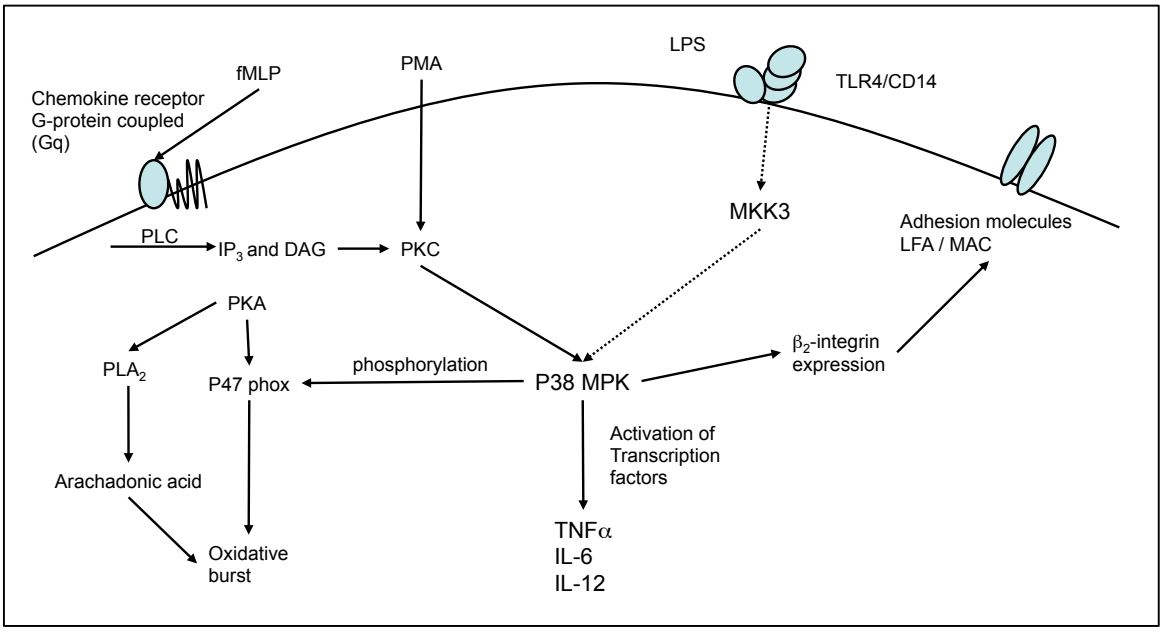


FIGURE 1-4 NEUTROPHIL PRIMING AND ACTIVATION OF P38-MITOGEN ACTIVATED PROTEIN KINASE (P38-MAPK) THROUGH CHEMOKINE AND TOLL-LIKE RECEPTOR ACTIVATION.

fMLP activates G-protein coupled receptors and Ras-dependent kinase, which subsequently activates and phosphorylates Mitogen activated protein kinase kinase (MKK)-1 and -2, thus phosphorylating the mitogen activated protein kinases ERK, JNK and p38-MAPK. In addition fMLP can activate p38-MAPK through the activation of MKK3. PMA bypasses membrane receptors and directly activates PKC (protein kinase-C) by mimicking DAG (diacyl-glycerol). PMA stimulation markedly increases neutrophil oxidative burst by activating NADPH-oxidase through redistribution of PKC and phosphorylation of protein 47-phagocyte oxidase (p47 ^{phox}).

1.8 Neutrophil chemotaxis

Neutrophils are rapidly recruited to sites of infection / inflammation through a process termed 'chemotaxis'. Initial tissue damage or infection is detected by resident sentinel cells, these include macrophages and mast cells, or by release of DAMPs from stromal cells. (Sadik et al., 2011) A huge variety of PAMP and DAMP molecules act on macrophages stimulating the release of pro-inflammatory mediators such as IL-1β, IL-6 and tumour-necrosis factor-α (TNFα) as well as chemokines. (Zeytun et al., 2010) Chemokines are small polypeptide cytokines released from cells that can induce the migration of neutrophils down a concentration gradient from a low to high concentration of the molecule. (Mollnes et al., 2002) Common macrophage-derived chemokines include the CXC chemokines (CXCL1-7), IL-8 (CXCL8) and lipid mediators (Leukotriene B4 [LT-B4] and platelet activating factor [PAF]). Other chemotactic molecules include bacterial derived proteins such as fMLP and activated complement proteins (e.g. C5a). The major function of chemokines is to prime neutrophils via G-protein coupled receptors in a process termed 'stimulus-response coupling' leading to activation of trimeric G-proteins, followed by increased intracellular Ca^{2+} levels, lipid remodelling and protein kinase activation. (Dale et al., 2008) The initial result of 'stimulus-response coupling' is neutrophil attachment and subsequent activation of the actin assembly leading to cytoskeletal rearrangement and polarisation of the cell with subsequent locomotion and migration down a chemokine concentration gradient to sites of infection or tissue injury. (Zigmond, 1977, Stossel, 1993) Secondary stimulation by chemokines can induce neutrophil degranulation and respiratory burst. Following chemokine receptor activation, downstream effector molecules such as the GTPases Rac1 and Rac2 (both members of the Rho family) (Bokoch, 1995) are involved in phagocyte migration and respiratory burst. Full activation of Rac is required for NADPH oxidase activity whereas partial activation is sufficient for neutrophil chemotaxis. (Hoppe and Swanson, 2004) Full activation requires further activation of the tyrosine kinases of the Src and spleen tyrosine kinase (Syc) family. In addition, Rac activation can lead to phosphorylation of ERK 1/2, p38-MAPK and JNK. (Benard et al., 1999)

Chemokines have important direct and indirect effects on neutrophil endothelial capture and adherence. Chemokines produced from the site of infection are taken-up and transported to the luminal surface of the endothelial cell, where they

are bound by heparan sulphate glycosaminoglycans, and are displayed here at high concentrations. Chemokines can therefore come into close contact with rolling neutrophils and bind to specific chemokine receptors on their surface. Chemokine receptor signalling stimulates neutrophils to rapidly upregulate and increases the avidity of β2-integrins for the endothelial ligand intercellular adhesion molecule 1 (ICAM-1) and induces clustering of this molecule to firm up endothelial adhesion; so called "inside-out signalling". The overall effect is therefore a firming-up of integrin-mediated binding of the neutrophil to the endothelial surface. The endothelial expression of ICAM-1 itself being upregulated by the pro-inflammatory cytokines TNFα and interleukin-1, and PAMPs released from sites of microbial infection. The net result of neutrophil and endothelial upregulation is the firm adhesion of neutrophils to the endothelium and cytoskeletal re-arrangement leading to the acquisition of a characteristic asymmetric shape with an advancing pseudopodium. (Hirsch, 1962)

1.9 Neutrophil recruitment across the vascular endothelium

Leukocyte recruitment occurs in a targeted multi-stage process initially involving interaction and adherence to vascular endothelium activated by pro-inflammatory mediators in the region of the post-capillary venule. Following 'activation', neutrophils become highly metabolically active resulting in cytoskeletal rearrangement and penetration of the endothelial cell layer (diapedesis) into the extra-cellular matrix. Defects in leucocyte adhesion result in recurrent bacterial infection and can be lethal. Two leucocyte adhesion deficiency syndromes are described in humans; leukocyte adhesion deficiency-type 1 (LAD1) and leukocyte adhesion deficiency-type 2 (LAD2). LAD1 is a rare autosomal recessive disorder characterised by the absence or reduced expression of the common β subunit (CD18) of the integrin receptor molecules LFA-1 and Macrophage-1 (Anderson and Springer, 1987). LAD2 is a deficiency in the carbohydrate structure Sialyl-Lewis X that renders neutrophils unable to adhere to E-selectin on activated endothelial cells (Etzioni et al., 1992). Conversely, inhibition of neutrophil recruitment can have a positive effect on ischaemia-reperfusion injury (Singbartl et al., 2000), sterile inflammation (Zarbock et al., 2006), and autoimmune disease (Chiriac et al., 2007).

1.10 Tissue adherence to vascular endothelium

Neutrophils continuously engage in weak physical interactions with the vascular endothelium probing for evidence of endothelial activation by brief binding events leading to cells rolling along the vessel wall. The molecules involved in this process are the selectins, integrins and their respective ligands, which are constitutively expressed on neutrophils and on vascular endothelial cells.

In flow-chambers, neutrophils are seen to roll along endothelial surfaces following numerous rapid on-off interactions mediated by selectins and their ligands. This transient rolling slows down the neutrophil and allows brief interrogation of the endothelial cell surface for chemokine expression such as IL-8 and PAF. Selectin adhesion is greatly enhanced following-activation of the endothelium by the pro-inflammatory cytokines TNFα, IL-1 and IFNγ found at sites of inflammation. Following further signalling events integrin-endothelial interactions mediate firm adhesion or "arrest" of neutrophils. Chemokine signalling induces changes in neutrophil receptor expression resulting in the shedding of L-selectin and up-regulation of the β2-integrin CD11b. The neutrophil adhesion cascade of tissue recruitment is therefore a multi-staged process of slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane. (Ley et al., 2007) Figure 1-5 summarises neutrophil recruitment across the vascular endothelium.

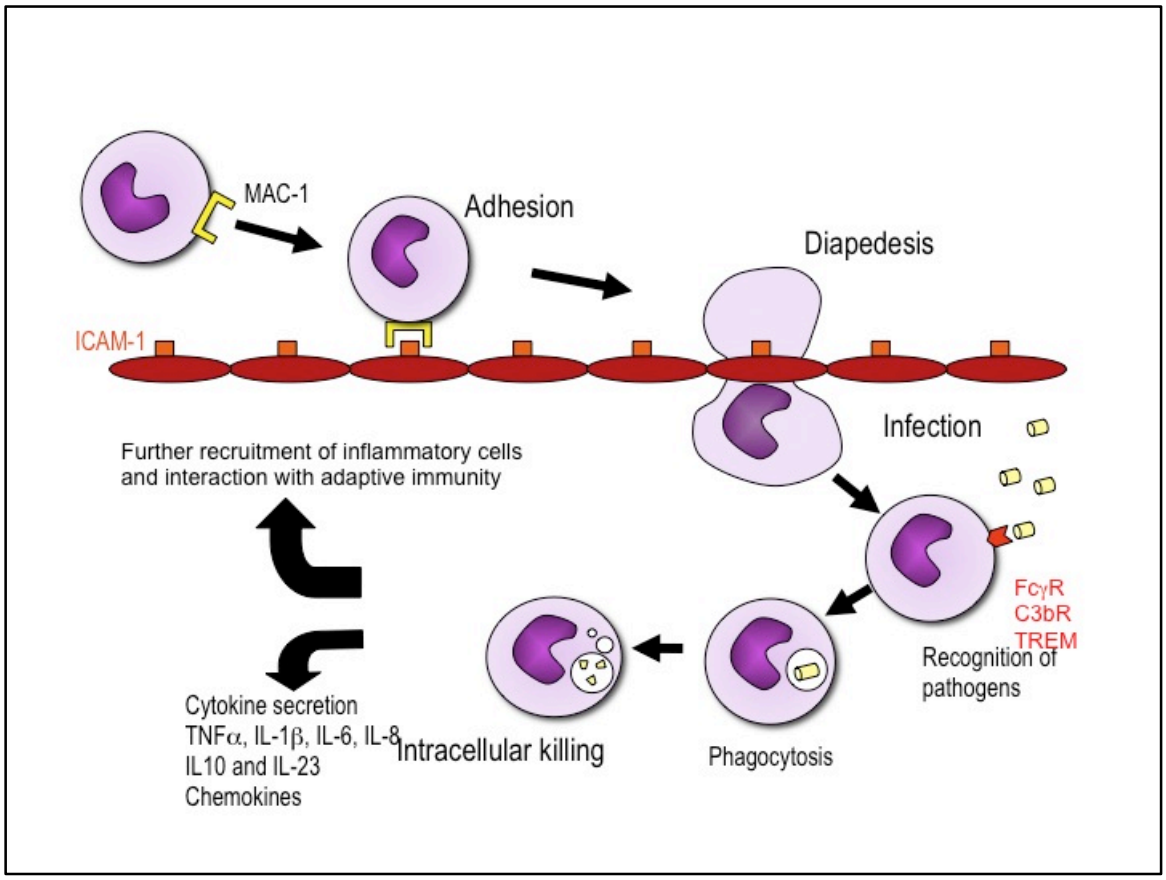


FIGURE 1-5 NEUTROPHIL RECRUITMENT ACROSS THE VASCULAR ENDOTHELIUM. (COURTESY OF DR R.D. ABELES)

1.11 Selectins

Selectins and their receptors mediate the initial low-affinity tethering of neutrophils to the endothelium; these interactions are easily disrupted by the sheer flow of blood (Kansas, 1996). L-selectin (CD62L) is constitutively expressed on all microvilli of circulating neutrophils. The molecule is a type-I transmembrane glycoprotein with an N-terminal calcium dependent "C-type" lectin domain and highly conserved cytoplasmic tail. (Zarbock and Ley, 2008) L-selectin interacts with sialylated oligosaccharide moieties on endothelial cell surface glycoproteins typically CD34, interestingly not present on liver sinusoidal endothelial cells (LSEC). The level of surface expression, cytoskeletal anchoring, and the distribution of L-selectin determine the tethering efficiency and rolling velocity (Dwir et al., 2001, Pavalko et al., 1995, Kansas et al., 1993). Cross-linking of L-selectin on neutrophils can also initiate β_2-integrin activation (Simon et al., 2000) but L-selectin-dependent rolling

alone does not induce neutrophil arrest (Lawrence et al., 1995). L-selectin expression on neutrophils can be rapidly down-regulated by proteolytic cleavage of L-selectin near the cell surface by the 'shedase' ADAM-17 (Smalley and Ley). The reduced surface expression of L-selectin observed in neutrophils from patients with sepsis is thought to occur through this mechanism. Blocking of L-selectin shedding *in vivo* increases signal input through L-selectin and results in an increase in neutrophil arrest, reducing rolling velocity (Hafezi-Moghadam and Ley, 1999). It is unclear if this is mediated directly through increased signalling and L-selectin activation or by increasing endothelial adherence.

The cytoplasmic tail of L-selectin is associated with calmodulin, α-actinin, both actin-binding proteins, and proteins of the ezrin-radixin-moesin (ERM) family. The cytoplasmic tail interacts directly with α-actinin anchoring L-selectin directly to the cytoskeleton, allowing L-selectin activation to facilitate neutrophil rolling and tethering. (Dwir et al., 2001) The ERM family proteins moesin and ezrin are involved in L-selectin expression on microvilli, tethering efficiency, and L-selectin shedding. (Ivetic et al., 2004) Activation of L-selectin through cross-linking leads to release of calmodulin and causes the concomitant shedding of L-selectin by conformational changes allowing access to cleavage sites. L-selectin cross-linking induces increased intracellular Ca^{2+} levels and Src-kinase-dependent tyrosine phosphorylation of the cytoplasmic tail resulting in activation of down-stream signalling pathways including Ras and p38-MAPK (Brenner et al., 1996, Waddell et al., 1995).

P-selectin (CD62P) and E-selectin (CD62E) are expressed on endothelial cells at sites of infection and both recognise specific neutrophil carbohydrate motifs (e.g. Sialyl-Lewis X) mediating endothelial tethering and rolling of neutrophils. Constitutive endothelial expression of P-selectin is low, however following activation by the pro-inflammatory cytokines IL-4 and IL-13; P-selectin is rapidly translocated from the Weibel-Palade bodies to the luminal endothelial surface. (Woltmann et al., 2000) P-selectin is also expressed on activated platelets but interestingly, not by LSEC. (Lopez et al., 1999) E-selectin is expressed exclusively by cytokine-activated endothelial cells and recognises glycoprotein (Sialyl-Lewis X) motifs on granulocytes, monocytes and T cells, and is important in neutrophil migration.

1.12 P-selectin glycoprotein ligand

P-selectin glycoprotein ligand (PSGL-1) [CD162] is highly expressed on the surface of neutrophils and appears to be the dominant neutrophil receptor for all 3 selectins. PSGL-1 binds selectin molecules through oligosaccharide moieties such as the Sialyl Lewis X–containing O-glycan and tyrosine sulphate residues (McEver and Cummings, 1997).

Binding of PSGL-1 to L-selectin leads to the formation of neutrophil aggregates through cell-cell interactions (Guyer et al., 1996). Physiologically selectins bind to their ligands with a remarkably fast on-off rate (Alon et al., 1995) under the presence of sheer-stress which supports adhesion, facilitating slow rolling and adhesion to the vascular endothelium. (Marshall et al., 2003) Antibodies targeted against these residues inhibit rolling velocity *in vivo* (Norman et al., 1995). Upon binding PSGL-1 initiates intracellular signalling events resulting in activation of β2-integrins (Simon et al., 2000), tyrosine phosphorylation (Hidari et al., 1997), secretion of cytokines (Hidari et al., 1997), transcriptional activation (Urzainqui et al., 2002) and cytoskeletal rearrangements (Ba et al., 2005). The cytoskeletal tail of PSGL-1, like L-selectin, is associated with ERM family proteins which mediate the formation of protrusive plasma membrane structures (Bretscher et al., 2000). Disruption of these proteins is associated with decreased rolling and adherence of neutrophils (Snapp et al., 2002). ERM can also activate Syk kinase, which is involved in PSGL-1-dependent signalling and regulates rolling. Blockade of p38-MAPK increases rolling velocity and reduces the number of neutrophil-interacting cells with E-selectin and ICAM-1 (Simon et al., 2000). PSGL-1 expression is down-regulated by endotoxin *in vivo* (Marsik et al., 2004) and by G-CSF but not dexamethasone, (Jilma et al., 2002) which may result in leucocytosis due to decreased adhesion to P-selectin in the BM venules. Dexamethasone and G-CSF both down-regulate L-selectin expression but only after a lag of 2 days.

1.12.1 Integrins

The β-integrins are the second class of adhesion molecules and are responsible for slow rolling, Velcro-like firm adhesion, post-adhesion strengthening, neutrophil migration, respiratory burst, phagocytosis, cell-pathogen interaction, cell-cell and cell-

extracellular matrix interactions. (Mayadas and Cullere, 2005) Integrins are present in an inactive state on circulating neutrophils.

Two subfamilies have been characterised. The molecules are heterodimers composed of one alpha-chain and a common beta-chain that defines the respective family. β1-integrins share the common beta-chain CD29. β1-integrins are not usually expressed on the surface of neutrophils, however, low levels of very-late antigen-4 (VLA-4), a heterodimer of CD29 and the alpha chain CD49d is expressed on up to 30% of neutrophils in sepsis. (Ibbotson et al., 2001) VLA-4 binds to the vascular cell adhesion molecule-1 (VCAM-1 or CD106), which is expressed on endothelium activated by pro-inflammatory cytokines. The exact contribution of VLA-4 to neutrophil recruitment in sepsis is uncertain but antibody-mediated blocking of L-selectin and β2-integrins does not ablate neutrophil adhesion in neutrophils from septic patients. (Brown et al., 2001) The β2-integrins appear to be the principle molecules involved in the 'arrest' of neutrophils on the vascular endothelium and subsequent migration therefore we will focus on these molecules.

1.12.2 β2-Intergrin: Leucocyte function antigen-1 (CD18/CD11a)

The leucocyte function antigen-1 (LFA-1) comprises the common β-chain CD18 and the CD11a alpha chain, and is thought to be the principle B_2-integrin involved in neutrophil adhesion and migration. It is expressed constitutively on the neutrophil surface and levels are unaltered by neutrophil activation during sepsis. The extracellular domain of LFA-1 contains a clever knee or "genu" which is present in a compact conformation with the legs of the molecule bent down towards the cell membrane in the resting state therefore concealing the ligand binding sites. Inside-out signalling generated by selectin-ligand binding and activation by cytokines (e.g. TNFα) and the chemokine CXCL8 results in this genu section extending in a two-step process to first a low-affinity state (during rolling) and then a high-affinity state (during arrest), where the ligand binding site is projected away from the membrane in a "clasp knife" action. (Takagi et al., 2002) This leads to an enhanced affinity of LFA-1 to its ligand on the endothelial cell, intracellular-adhesion molecule-1 (ICAM-1 or CD54). ICAM-1 expression on endothelial cells is upregulated by the pro-inflammatory cytokines TNFα and IL-1 secreted by macrophages. (Yang et al., 2005)

Additionally clustering of LFA-1 occurs due to lateral mobility of the molecule at areas where cell-surface ligands are present, which increases cell avidity. During neutrophil migration LFA-1 forms ring-like clusters at the neutrophil-endothelial junction. (Shaw et al., 2004) These clusters move from the leading edge to the rear of the neutrophil (uropod), always in contact with their major ligand ICAM-1. Binding of integrins to their ligands induces outside-in signalling with concomitant activation of several intracellular signalling pathways. (Zarbock and Ley, 2008)

1.12.3 β2-Intergrin: Macrophage-1 (CD18/CD11b)

The β2-integrin, comprised of CD18 and alpha chains CD11b, is termed macrophage-1 (Mac-1) and neutrophils have constitutive low-level surface expression in the resting state. However, Mac-1 is contained in secretory vesicles as well as secondary and tertiary granules; these fuse with the plasmalemma upon neutrophil activation. CD11b expression can therefore be used as a marker of degranulation and in sepsis Mac-1 expression is seen to be upregulated in most studies. Mac-1 recognises the ligand for β2-integrin ICAM-1, expressed on endothelial cells and regulates rolling velocity of neutrophils *in vivo*. (Diamond et al., 1990) Mac-1 however binds a number of additional ligands, including bacterial and fungal glycoproteins, heparin, coagulation factor Xa, fibrinogen and complement protein C3bi. (Altieri et al., 1988, Beller et al., 1982) In addition Mac-1 is involved in neutrophil-platelet interactions and intravascular crawling of neutrophils. Mac-1 is also critical for complement-mediated phagocytosis. Mac-1 cooperates functionally with other ligands including Fragment crystallisable-γ receptors (FcγR), TLRs and CD14. (Sabroe et al., 2003) In addition, activation of Mac-1 modulates neutrophil apoptosis. (Mayadas and Cullere, 2005) Neutrophil adhesion to Mac-1 ligands, fibrinogen and ICAM-1, extends the life span of neutrophils. This is mediated through activation of a Serine/Threonine specific protein kinase called Akt [also known as Protein kinase B], MKK activation and NF-κB activation. (Whitlock et al., 2000) Soluble fibrinogen can activate neutrophils and delay apoptosis through Mac-1 activation so adhesion is not essential to generate survival signals. (Rubel et al., 2003)

1.13 Neutrophil transmigration

Upon receiving secondary chemotactic signals adherent leucocytes proceed to migrate through inter-endothelial spaces down a chemokine gradient. (Cinamon et al., 2004) Neutrophils initially crawl along blood vessel walls in a Mac-1-and ICAM-1 dependent manner, seeking optimal sites for transmigration. (Phillipson et al., 2006) The route of transmigration, either paracellular or transcellular, may depend upon the composition of the endothelium, basement membrane, and the presence of pericytes which varies depending on the vascular bed encountered. After chemokine activation adherent neutrophils can induce 'docking structures' to form on endothelial cells. (Carman and Springer, 2004) These structures are endothelial projections rich in ICAM-1 and cytoplasmic molecules such as ERM proteins and cytoskeletal components such as viniculin, α-actinin and talin-1. (Barreiro et al., 2002) The development of these docking sites is dependent on integrin-ligand binding, which induces clustering in association with cytokine proteins dependent on the expression of phosphatidylinositol-4-5-bisphosphonate and Rho family GTPase. (Carman and Springer, 2004, Barreiro et al., 2002) These docking structures are seen to develop in projections that may initiate transendothelial cell migration.

Transmigration is poorly understood, however, activation of p38-MAPK and ERK1/2 in the endothelium appears to be essential for neutrophils to transverse the endothelial barrier (Stein et al., 2003). Ultimately neutrophils enter the extra-cellular space and accumulate in and around the infectious microbe and damaged cell.

Specificity exists in that neutrophil migration relies on LFA-1 – ICAM-1 interactions in combination with the chemokine receptors CXCR1 and CXCR2 binding the chemokine IL-8. This differs from monocytes which utilise VLA-4 – VCAM-1 interactions together with the chemokine CCL2 binding to the chemokine receptor CCR2. Temporally distinct expression of adhesion molecules and chemokines at infectious sites typically results in early neutrophil recruitment (hours to days) followed later by monocyte recruitment (days to weeks). In sepsis, transmigration appears to be impaired and affected by a number of circulating factors, such as IL-8, C5a and TNFα resulting in extensive neutrophil-endothelial interactions and potentially enhancing vascular damage. TNFα in addition inhibits apoptosis and enhances production of ROS but suppresses CXCR2 expression. (Asagoe et al., 1998)

1.14 Neutrophil recruitment into the liver and liver sinusoidal endothelial cell: does it differ?

In response to acute liver injury neutrophils marginate within the hepatic sinusoids and post-sinusoidal venules prior to transmigration into the tissue. This accumulation is mediated by a variety of pro-inflammatory cytokines, such as IL-1 and TNFα, and a number of chemokines including IL-8, MIP-2, KC, cytokine-induced neutrophil chemoattractant-1 (CINC-1), PAF and ostopontin-1 (localised within the biliary epithelial cell). (Ramaiah and Jaeschke, 2007) In contrast to adhesion in post-sinusoidal-venules neutrophil accumulation within sinusoids does not appear to be dependent on selectins and β2-integrins. (Jaeschke, 2000) LSEC do not express CD34 the receptor for L-selectin and P-selectin is deficient in the liver. Neutrophil accumulation within sinusoids and post-sinusoid venules does not typically cause liver damage *per se* without extravasation into the parenchyma. (Chosay et al., 1997)

Increased production of CXC chemokines within hepatocytes can cause neutrophil infiltration, extravasation and consequent neutrophil-dependent cytotoxicity, but this is not always the case. There appears to be a need to establish a chemotactic gradient, at the right time and in sufficient quantities. (Dorman et al., 2005) Apoptotic hepatocytes can mediate neutrophil extravasation and can generate CXC chemokines in sufficient quantities to trigger neutrophil accumulation into the liver in the presence of other potent pro-inflammatory mediators (chemokine in isolation have little direct effect). (Jaeschke et al., 1998) Direct-cell contact through endothelial gaps with either apoptotic or necrotic cells may induce extravasation. ROS and lipid peroxidation products including lipid aldehydes are potent chemoattractant factors for neutrophils. (Jaeschke, 2000) Activated neutrophils can recruit more neutrophils by their production of LT-B4, as seen during ischaemia-reperfusion injury. (Jaeschke, 2003)

1.15 Phagocytosis

Phagocytosis is defined as the vesicular internalisation of solids such as bacteria by the extension of pseudopods around the particle involving actin filaments (Bajno et al., 2000) and is central to the microbicidal function of neutrophils. Pathogens and

foreign debris are first engulfed into a plasma membrane-derived "digestive vacuole" or phagosome; (Cohn and Hirsch, 1960) this subsequently acquires anti-microbial properties through granule fusion in a process termed 'maturation'. (Lee et al., 2003) Neutrophils from sepsis patients show enhanced internalisation and destruction of micro-organisms. (Martins et al., 2003)

Neutrophils can phagocytose both opsonised and non-opsonised particles. Phagocytosis is initiated following recognition of particles by a number of PRRs; these include scavenger receptors (e.g. TREM), complement (e.g. Mac-1) and immunoglobulin receptors (e.g. FcγR). There are 3 types of Fc receptor produced by neutrophils and the pattern of surface expression depends on the state of activation. Resting neutrophils express the low affinity FcγRIIA (CD32) and FcγRIIIb (CD16) and following priming with IFN, neutrophils express the high affinity FcγRII (CD64). (McKenzie and Schreiber, 1998) The importance of immunoglobulin opsonisation in neutrophil uptake and destruction is highlighted by X-linked agammaglobulinaemia (Bruton's disease) where there is defective class switching by B-cells preventing gamma-globulin production. This results in a neutrophil defect with the failure of phagocytes to clear bacterial pathogens from infected tissues and blood. (Bruton et al., 1952) The response induced by the neutrophil depends on the ligand receptor response. Complement opsonised particles are internalised by gently sinking into the cell, whereas FcγR ligation initiates the rapid extension of pseudopods that envelop around the opsonised microbe. (Greenberg and Grinstein, 2002)

When invading microbes come into contact with neutrophils binding occurs to multiple ligands on the cell surface. The binding of opsonised microbes to FcγR on the neutrophil surface leads to clustering of the transmembrane FcγR and subsequent recruitment of Src-kinases to the clustered area. (Kiefer et al., 1998) These Src-kinases activate the FcγR through phosphorylation of the tandem tyrosine residues within an area termed the immunoreceptor tyrosine activation motif (ITAM). (Greenberg and Grinstein, 2002) Phosphorylation of the ITAM generates docking sites for proteins bearing a SH_2- domain especially the Syk kinases. This is an essential step as Syk-deficient mice are unable to ingest IgG-opsonised particles. (Botelho et al., 2000) Downstream activation of protein and lipid kinases then occurs especially Phosphatidylinositide 3-kinase (PI3K) and Phospholipase C (PLC). (Cox and Greenberg, 2001) These induce the polymerisation of actin and localise

membrane remodelling required for particle ingestion. Cessation of PI3K is abrupt and in part is due to the recruitment of lipid phosphatase to the phagocytic cup. (Cox et al., 2001) Diacylglycerol (DAG) is also produced through the action of PLC on phosphatidylinositol 4,5-bisphosphate. DAG activates protein kinase C (PKC) that can participate in particle uptake. (Botelho et al., 2000) Differences are seen in the molecules recruited, which may account for the different responses seen with complement and immunoglobulin mediated phagocytosis such as MEK-1, which appears to be selectively involved in FcγR mediated phagocytosis in neutrophils. (Mansfield et al., 2000)

Once formed, phagosomes enter the endocytic pathway and undergo a rapid sequence of events leading to maturation. Termed the 'kiss and run' hypothesis, sequential granule fusion occurs altering both the contents and membrane composition of the phagosome. (Desjardins, 1995) Maturation is an energy dependent process resulting in vacuolar remodelling and fusion of cytoplasmic granules. Through this process the phagosome acquires a highly toxic environment including the acquisition of microbial enzymes, vacuolar (V) ATPase and the NADPH oxidase complex. (Kinchen and Ravichandran, 2008) The dynamics of this process are rapid with phagocytosis of opsonised particles and subsequent phagosome maturation occurring in as little as 20 seconds. (Segal et al., 1980)

There appears to be a hierarchy of microbial susceptibility to phagocyte-mediated death from highly susceptible organisms such as *streptococci* and yeast to microorganisms that have adapted to survive the harsh environment of the phagosome such as *mycobacteria* that survive by suppressing calcium influx into the cell. Others such as *listeria* evade phagocytes using the actin assembly of host cells to directly spread cell to cell. (Zhang et al., 2009, Flannagan et al., 2009)

1.16 Neutrophil granules

Neutrophils are characterised morphologically by their large quantity of poorly staining cytoplasmic granules. The granules play an important part in the process of microbial killing. Neutrophils exit the BM after going through a prolonged development process the purpose of which is to equip them with a complete array of pre-packaged antimicrobial enzymes and sufficient surface receptor expression with more in reserve for rapid up-regulation allowing them to operate independently of

protein transcription and translation in the initial phase. The different content of granules is explained by the 'targeting-by-timing' hypothesis whereby granule production occurs continuously during development from the promyelocyte to the segmented neutrophil but due to the changing profile of granule proteins synthesised during different stages granule content differs. Azurophilic granules are synthesised at the promyelocytic stage, specific granules at the myelocytic stage and gelatinase granules at the promyelocytic and band cell stage. However, to some extent this should be thought of as a continuum with subsets being identified due to the presence of an altering array of marker proteins. (Borregaard et al., 2007) Secretory vesicles are endocytotic particles. Neutrophil granules have been recognised since Metchnikov and Ehrlich. Further work performed in 1952 by Chediak, a Cuban physician, uncovered the antimicrobial importance of these granules. Chediak described patients with a rare autosomal recessive condition with several distinctive characteristics including recurrent pyogenic infections, partial albinism and peripheral neuropathy associated with giant cytoplasmic leukocyte granules. (Chediak, 1952) Chediak-Heigasi syndrome, as it is eponymously called, results from the defective gene CHS1/LYST, a gene belonging to the BEACH family of vesicle trafficking regulatory proteins. (Zarzour et al., 2005) The defective protein prevents the formation of the phagolysosome and failure of degranulation into the phagosome, cytoplasmic granules then become excessively enlarged in the neutrophil cytoplasm, and ultimately this results in impaired neutrophil bacteriolysis. Affected children suffer recurrent infections and the syndrome is associated with oculocutaneous albinism.

Different subsets of granules have now been characterised by phase contrast electron microscopy (Bainton, 1993), flow cytometry and functional analysis. Neutrophil granules develop during the promyelocytic and myelocytic phases of development in the BM, and their contents depend on the proteins being synthesised at the time and the presence of signalling peptides. The different granules have long been recognised to possess microbicidal activity and contain reservoirs of digestive and hydrolytic enzymes as well as the components of the NADPH oxidase. (Faurschou and Borregaard, 2003)

The cytoplasm contains four differing types of granules that bud from the Golgi network in a sequential fashion during neutrophil maturation in the BM, resulting in a difference in granule contents and membrane structure [see figure 1-6]. (Borregaard et al., 2007)

1.16.1 Azurophilic granules

Azurophilic or primary granules are the first to appear during neutrophil development. They contain a vast array of antimicrobial factors including myeloperoxidase (MPO), phospholipase-A2 and three predominant neutral proteases: cathepsin G, elastase and proteinase-3. (Bokoch, 1995) In addition they contain antibacterial proteins including lysozyme (approximately one third of the cellular contents), defensins, (Ganz, 2003) cathelicidins (Ding et al., 1996) and bacterial/permeability-increasing protein (BPI). BPI is a membrane bound protein within granules that can bind and neutralise LPS, whilst also possessing the ability to permeabilise and neutralise *E.coli*. (Weiss et al., 1978) Azurophilic granules can be identified on flow cytometry due to the characteristic presence of surface CD63. (Niessen and Verhoeven, 1992) The granular contents are enmeshed within an extensive matrix composed of negatively charged sulphated proteoglycans. (Ptasznik et al., 1996) This matrix sequesters the mainly cationic proteins and together with the acidic environment ensures the enzymes are kept inactive. Interestingly, the absence of neutrophil elastase is the cause for cyclical and severe congenital neutropenia (Dale et al., 2000).

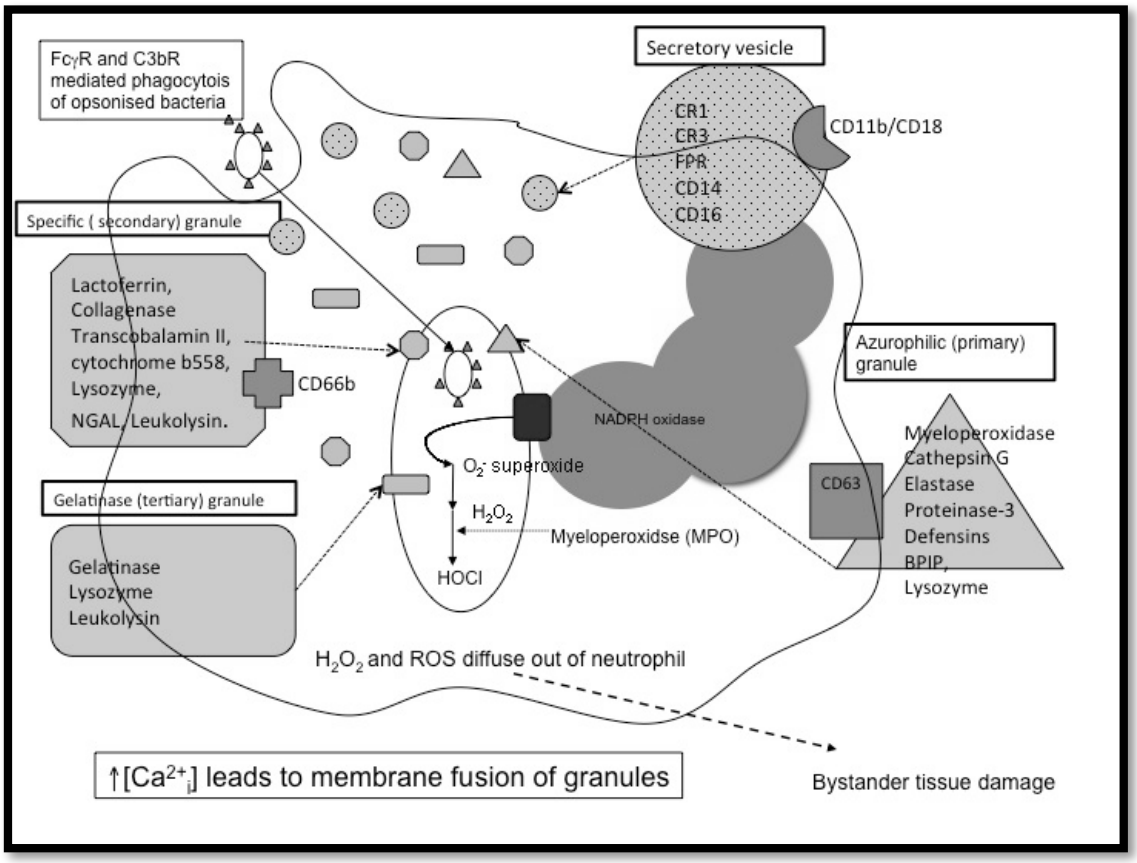


FIGURE 1-6 NEUTROPHIL GRANULES AND SECRETORY VESICLES CONTENT AND SURFACE MARKERS INCLUDING RELATIONSHIP TO OXIDATIVE BURST AND MICROBIAL EXTERMINATION IN THE PHAGOSOME.

Abbreviations: BPIP – Bacterial permeability increasing protein.

1.16.2 Specific granules

Specific or secondary granules characteristically express membrane-bound CD66b allowing identification by flow cytometry. The granules contain unsaturated lactoferrin (able to sequester iron and copper), transcobalamin II and lysozyme (approximately two thirds of the cellular contents), neutrophil gelatinase-associated lipocalin (NGAL) and gelatinase, (Bokoch, 1995, Bundgaard et al., 1994) and a neutrophil specific matrix metalloproteinase (MMP) leukolysin component. Alexander Fleming discovered lysozyme in 1921; he demonstrated that his own nasal mucous could kill bacteria after ‘sneezing’ over a culture dish containing bacteria. Lysozyme was later shown to act by disrupting the bacterial peptidoglycan cell wall leading to cell lysis.

In addition, specific granules possess membrane bound flavocytochrome b_{558}, the core component of the NADPH oxidase, which when assembled on the phagosome membrane acts as an electron channel. (Segal and Jones, 1979) The importance of specific granules is highlighted by the rare congenital absence of specific granules caused by the functional loss of transcription factor C/EBPε or Gfi-I. This condition is associated with recurrent infections with *Staphylococcus aureus* and *Pseudomonas.spp*. (Lekstrom-Himes et al., 1999)

1.16.3 Gelatinase granules

Gelatinase or tertiary granules, which lack CD66b, are typified by high concentrations of gelatinase in the absence of lactoferrin and are likely to represent a continuum of specific granules. (Bokoch, 1995) Lysozyme is present in gelatinase granules but less than that present in azurophilic or specific granules.

1.16.4 Secretory vesicles

Secretory vesicles characteristically contain albumin and express surface alkaline phosphatase and CD35. (Lee et al., 2003) These empty vacuoles are endocytotic in origin and serve as a reservoir of membrane components, such as CD11b. (Segal, 2005) Secretory vesicles are the first to be mobilised during neutrophil priming, rapidly fusing with the plasma membrane following the binding of selectins to PSGL-1. (McEver and Cummings, 1997) This is supported by studies showing the absence of secretory vesicles following neutrophil migration through a skin window chamber (Ptasznik et al., 1995). This mechanism is thought to underlie the upregulation of CD11b observed on neutrophils isolated from patients with sepsis.

1.16.5 Lysosomes

Lysosomes contain acid hydrolases and therefore perform similar duties to azurophilic granules but fuse with the phagosome at a later time point so are considered separate.

1.16.6 Granules and phagosome maturation

Granular fusion with the phagosome and plasma membrane occurs in an elegant sequential fashion with increasing intracellular calcium [Ca^{2+}]i levels within the neutrophil appearing to be the critical signalling event leading to granule fusion. (Stendahl et al., 1994) Granules possess different calcium thresholds, a characteristic feature that appears to be regulated by differential granular expression of a family of calcium responsive proteins called the synaptotagmins. (Lindmark et al., 2002) Secretory vesicles have the lowest threshold and azurophilic the highest. (Sengelov et al., 1993) The result is that secretory vesicles can fuse rapidly with the plasma membrane following initial neutrophil priming leading to up-regulation in expression of surface receptors such as CD11b. Sequentially higher thresholds of [Ca^{2+}]i are required for gelatinase, specific and finally azurophilic granules to fuse with the plasma membrane or phagosome (azurophilic granules principally fuse with the phagosome membrane).

In order to facilitate granular fusion with the phagosome there is an associated calcium-dependent disassembly of the actin coat surrounding the phagosome-cup, revealing vesicle docking and fusion sites. (Zeytun et al., 2010) As the phagosome matures further it acquires calmodulin in a calcium-dependent fashion where it can catalyse lipid bilayer membrane fusion. (Peters and Mayer, 1998) Finally the calcium binding proteins Annexin I and III appear to associate with the matured phagosome although their function is as yet unclear. (Rosales and Ernst, 1997)

Activation of Hck, a tyrosine protein kinase belonging to the Src family of protein kinases, appears to be important in the process of phagosome maturation. Src family kinases are activated following the clustering of membrane receptors such as the FcγR. Hck is localised to the azurophilic granules and upon phagocytosis of opsonised particles it activates and translocates towards the phagosome. The important role for Hck is supported by the finding that neutrophil internalisation of mycobacteria fails to activate Hck, leading to the failure of azurophilic granules to fuse with the phagosome while fusion of secretory vesicles is unaffected. (N'Diaye et al., 1998) Overall, comparatively little is known about the events that trigger membrane fusion during maturation of phagosomes in neutrophils.

Interestingly, different stimuli can cause variability in the location of fusion events. Soluble agonists, such as PMA, cause secretory vesicles and granules to fuse

throughout the plasma membrane; however, during phagocytosis fusion is restricted to the nascent and formed phagosome. Specificity for fusion events appears to involve soluble N-ethylmaleimide-sensitive-fusion protein attachment protein receptors (SNARE), donor, and receptor SNARE molecules e.g. SNAP-23 on specific and tertiary granules which interact with synaxin-6 on the phagosome (terahelical complex). (Peters and Mayer, 1998)

Phagosome maturation therefore involves a complex sequence of events initially involving rapidly increasing $[Ca^{2+}]_I$ levels within the cell as a consequence of priming and activation signals. This results in the loss of the actin coat around the phagosome-cup followed by sequential fusion of the specific and then azurophilic granules with the now revealed docking sites. Some researchers dispute the relevance of the sequential fusion of granules with the phagosome as granular fusion events occur in a rapid fashion, (Segal et al., 1980) with the suggestion that sequential granular fusion events have relevance at the site of the plasma membrane during neutrophil priming but this is far from clear.

1.17 The Neutrophil NADPH oxidase

Neutrophils can produce large numbers of ROS generated almost exclusively from NADPH oxidase in a process termed oxidative burst (OB). (Sbarra and Karnovsky, 1959) Leucocyte-specific NADPH oxidase is a multi-subunit entity with membrane bound and soluble components that assembles into a heteromeric complex when the cells are stimulated. (Vignais, 2002) The importance of this step is highlighted by chronic granulomatous disease (CGD), a condition where OB is defective with cells being unable to generate superoxide. CGD is characterised by recurrent life-threatening bacterial and fungal infections along with granuloma formation. (Baehner and Nathan, 1967) Indeed, it is the study of this condition that has revealed much about the NADPH oxidase as a number of different defects can result in the condition; the most common defects occurring in the X-linked gene encoding the gp91 subunit of the flavocytochrome b_{558}. The NADPH oxidase requires assembly prior to activation within the phagosome with the intrinsic components of the NADPH oxidase being mostly absent from the nascent phagosome at rest. The catalytic core of the complex, the Haem containing flavocytochrome b_{558}, is bound to the internal membrane of specific granules, being delivered through fusion events during

phagosome maturation. The cytosolic components, termed 'phagocyte oxidase' (phox) proteins are translocated to the membrane-bound flavocytochrome b_{558} in activated phagocytes. The NADPH oxidase is a heterodimer composed of a larger glycoprotein: phagocyte oxidase glycoprotein 91 ($gp91^{phox}$), and a small protein: phagocyte oxidase protein 22 ($p2^{2phox}$). The C-terminus of the $gp91^{phox}$ participates in NADPH and flavin adenine dinucleotide (FAD) binding. The N-terminus of the $gp91^{phox}$ possesses a haem co-ordinating region, which binds two heam molecules either side of the plasma membrane consistent with the $gp91^{phox}$ function as an electron transporter. $p22^{phox}$ provides a high affinity binding site for cytosolic NADPH oxidase subunits. Associated proteins of cytosolic origin promote transition of flavocytochrome b_{558} from a resting to activated state. (Vignais, 2002) Three components $p47^{phox}$, $p67^{phox}$ and a small GTP protein, either Rac-1 or Rac-2, are sufficient, when used in a cell free assay, to activate the flavocytochrome b_{558} core. (Abo et al., 1992) In addition, $p40^{phox}$ appears to be associated with $p47^{phox}$ and $p67^{phox}$ and may play a role in regulation of NADPH-oxidase activity. (Wientjes et al., 1993) This process appears to involve phosphorylation of the cytosolic components and the involvement of phosphatidylinositol phosphates. $p47^{phox}$ is heavily phosphorylated during neutrophil activation and appears to be an adaptor molecule forming a bridge between $p22^{phox}$ and $p67^{phox}$. (Segal, 2005) Pentoxifylline attenuates activation of signaling molecules involved in activation of $p47^{phox}$ and suppress the subsequent assembly of the NADPH machinery through both PKA-dependent and PKA-independent mechanisms (Costantini et al., 2010). $p67^{phox}$ binds either Rac-1 or Rac-2 an essential requirement for NADPH activation. (Bokoch, 1995)

The assembled oxidase generates the superoxide anion by transfer of one electron from cytosolic NADPH to molecular oxygen to generate the superoxide anion ($\bullet O_2^-$). Superoxide is rapidly converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). Superoxide and H_2O_2 can interact to form the hydroxyl radical $\bullet OH$ via the Harber-weiss reaction, catalysed by heavy metals such as iron (Fenton reaction). The hydroxyl radical is highly toxic but there is some debate about its generation *in vivo* due to the presence of unsaturated lactoferrin in the granule protein, which inhibits this reaction. Myeloperoxidase is a di-haem containing heterodimer abundantly present in neutrophils composing 25% of azurophilic granular protein. Interestingly, the green tinge of secretions, such as pus

and mucous, rich in neutrophils is due to the haem molecules in MPO. MPO converts H_2O_2 and chloride anions into hypochlorous acid, which in combination with halides can produce hypochlorous acid. (Weiss et al., 1982) MPO-mediated halogenation is important in the antimicrobial action of neutrophils but there is some debate regarding the exact mechanism of action, with recent work supporting an alternative hypothesis whereby indirect activation of granule enzymes rather than direct microbial toxicity occurs. (Reeves et al., 2002) However, hypochlorous acid appears to be generated within the phagosome but the role of MPO may in addition be to preserve the function of granule proteins by preventing accumulation of H_2O_2. (Nauseef, 2007) The NADPH oxidase is more pronounced in neutrophils compared to macrophages. Indeed, much research has focused on the role of NADPH oxidase in microbial killing linked to the defects seen in CGD, whereas other anti-microbial mechanisms such as serine proteases and defensins have to some extent been neglected. More recently Segal and others have questioned the precise role of NADPH oxidase *in vivo* as many assays reported in the literature have been performed in a cell free environment and many of the free radical species may not form *in vivo*. (Segal, 2005) The exact location of the NADPH assembly may also be important as soluble agonists such as PMA can lead to assembly at the plasma membrane *in vitro*; however, assembly *in vivo* is thought to occur exclusively at the phagosome membrane. The presence of a number of heavy metal scavengers, such as lactoferrin, within the phagosome may prevent the formation of the hydroxyl radicle and subsequent hypochlorous acid. Indeed, MPO deficiency appears to lead to little clinical consequence (cf. CGD). It has therefore been postulated that the critical role of the NADPH oxidase is to dissolve the granule matrix and activate the enzymatic granular content, although this is a contentious area.

Granules are kept acidic (approximately pH 5 by the presence of a proton pump) in order to maintain the acidic glycoprotein matrix and keep the proteases in a quiescent state. The activity of NADPH oxidase alters the appearance of the phagocytic vacuole soon after the phagocytosis of bacteria, with loss of the ground glass appearance seen on electron microscopy. The change in appearance of the phagosome fails to occur in patients with CGD, suggesting that the oxidase changes granule contents. The NADPH oxidase transfers electrons into the phagosome depolarising the membrane but in order for continued function this charge must be compensated. Phagosomes briefly increase their pH shortly after granular fusion due

to the consumption of H+ ions in the dismutation of superoxide to H_2O_2. (Cech and Lehrer, 1984) K+ enters the cell as an inward rectifying current through a large conductance Ca^{2+}-activated K^+-channel. This neutralises the electrogenic displacement of electrons by the oxidase leading to a hypertonic milieu. The brief rise in vacuolar pH and hypertonic K^+ facilitate dissolution of the matrix glycoprotein, releasing and activating the cationic proteases into solution (Reeves et al., 2002). In order for these hypertonic conditions to develop, water penetration of the phagosome must be prevented and this is achieved by encasing the phagosome in a meshwork of cytoskeletal proteins, including paxillin and vinculin. (Granfeldt and Dahlgren, 2001)

1.18 Microbial killing in the phagosome

The critical steps in neutrophil killing appear to be phagocytosis followed by 'oxidative burst' and production of ROS through the actions of the NADPH oxidase with subsequent generation of hydrogen peroxide and 'hypochlorous acid' within the phagosome through the actions of SOD and MPO. Hypochlorous acid has been shown to cause membrane changes through chlorination of membrane proteins, ultimately leading to cell lysis. (Vissers et al., 1994) Defensins are a second neutrophil membrane disruption technique; these highly conserved peptides are widespread across animals and plants alike, and result in disruption of microbial membranes through binding and formation of a pore-like membrane defect [c.f. the membrane attack complex of complement]. (Ganz, 2003) Gene knockout mice show the importance of neutrophil granular enzymes in microbial defence. Neutrophil elastase (NE)-deficient mice are susceptible to gram-negative and candida infections. Cathepsin-G deficiency on its own produces a less severe defect with susceptibility to *Aspergillus fumigatus* and *Candida.spp*; however, deficiency of both enzymes causes a profound inability to kill phagocytosed bacteria similar to CGD despite preserved functioning of the NADPH oxidase and protein iodination. (Reeves et al., 2002)

1.19 Neutrophil cytokine production

Neutrophils are not classically credited with producing significant amounts of cytokines at sites of inflammation; this principally being thought of as the preserve of macrophages. Terminal differentiation of neutrophils was believed to preclude them

from undertaking further protein synthesis. However, recently it has been shown that neutrophils in peripheral tissues undergo active protein transcription and translation releasing chemokines, cytokines and lipid mediators. (Altstaedt et al., 1996, Cassatella, 1995) Although neutrophils produce much smaller quantities of inflammatory mediators than macrophages their sheer number mean they can contribute significantly to the recruitment of further neutrophils and other inflammatory cells, particularly monocytes and macrophages.

Following TLR activation primed neutrophils rapidly generate IL-8 in response to TNFα. (Zu et al., 1998) They also produce the anti-inflammatory IL-1R antagonist (Stanley et al., 1996) and the anti-inflammatory cytokine IL-10. (Zhang et al., 2009) There is contentious data regarding the ability of neutrophils to secrete IL-1β, IL-6 and TNFα (Kasama et al., 2005) with macrophage contamination cited as the explanation for this phenomenon. More recently however, intracellular cytokine staining techniques have demonstrated neutrophil production of IL-17A in an IL-23-dependent manner in response to *Aspergillus fumigatus* (Werner et al., 2011). Neutrophils also produce macrophage colony-stimulating factor attracting macrophages, suggesting they mediate transmission from acute inflammation into either resolution or chronic infection. (Soehnlein et al., 2009) This may lead to resolution of inflammation with progression to a macrophage predominant cellular infiltrate. It is increasingly recognised that neutrophil cytokine production appears to play a role in the development of chronic inflammatory conditions such as chronic obstructive pulmonary disease, arthritis and inflammatory bowel disease. (Quint and Wedzicha, 2007, Tanaka et al., 2006, Chin and Parkos, 2006) For example, in immune complex-mediated arthritis neutrophils located within the joint release chemokines and cytokines including LT-B4, MIP-1α, MIP-2 and IL-1β, which have direct and indirect activity encouraging further neutrophil recruitment. Secreted proteases can degrade or activate secreted chemokines and cytokines in the extra-cellular space thereby neutrophils can recruit further neutrophils.

1.20 Apoptosis and resolution of inflammation

Apoptosis or programmed cell death has a significant role in the control of the immune response. Apoptosis limits the potentially destructive effects of neutrophil

cytotoxicity and may prevent the survival of organisms that have adapted to the harsh environment within the neutrophil phagosome (e.g. mycobacteria). Neutrophils are cleared from tissues by apoptosis and are subsequently phagocytosed by resident macrophages, a process known as efferocytosis that promotes the resolution of the inflammatory process. Neutrophils are terminally differentiated cells and have a circulating life span of 5.4 days, undergoing constitutive apoptosis in the spleen and BM.

Apoptosis is an active process that can be accelerated or delayed by competing anti and pro-apoptotic signals. GM-CSF, G-CSF, IL-1, TNF, IL-6, LT-B4, C5a and LPS, all prolong the life span of circulating neutrophils. (El Kebir and Filep, 2013) Apoptosis is regulated at the transcription level. High doses of TNF or cellular stress can accelerate the spontaneous rate of apoptosis. Caspase 3 and 8 are probably involved in constitutive neutrophil apoptosis. Transcription regulation of apoptosis-related proteins by IFN consensus sequence-binding protein promotes cell death by up-regulating expression of a pro-apoptotic caspase 3 precursor, and down-regulates the anti-apoptotic protein Bcl-X. Other signalling pathways that promote *in vitro* survival in neutrophils include mitogen-activated protein-extracellular signal-regulated kinase Serine/Threonine kinase (MAPK-ERK) activation and NF-κB mediated transcription of survival proteins. Neutrophil adhesion to Mac-1 ligands, fibrinogen and ICAM-1, extend the life span of neutrophils. The delay in apoptosis in response to ICAM-1 adhesion is attributed to Akt activated through the PI-3K pathway. Soluble factors such as fibrinogen can promote neutrophil activation (increase $[Ca^{2+}]_i$ and Mac-1 expression) in a Mac-1 dependent manner and also delays apoptosis through the activation of NF-κB and MAPK-ERK. Therefore neutrophils do not need to be adherent to the endothelium for survival signals to be generated. Antibody cross-linking of Mac-1 in the presence of anti-Fas antibody or TNF alters this balance as activation of Akt is decreased. This leads to NADPH oxidase activation and release of ROS that leads to activation of SHIP (Src homology 2-containing inositol phosphatase), an inositol phosphatase that hydrolyses products of PI-3K through the Src kinase Lyn. Extracellular ROS may diffuse back into cells to signal death which may be relevant *in vivo* under conditions of high neutrophil accumulation. Mac-1 is also required for apoptosis induced by other cell surface receptors perhaps due to functional co-operation of Mac-1 with other receptors. For

example, TNF promotes neutrophil apoptosis in response to engagement of receptors for fMLP, immune complexes and Zymosan. The role of Mac-1 in apoptosis might be limited to promoting ROS generation, which in turn activates caspase 8 through receptor independent mechanisms.

Phagocytosis of complement-opsonised *E-coli* accelerates apoptosis and is termed phagocytosis-induced cell death (PICD). A certain threshold of ROS generation is required and a sustained intracellular oxidative burst appears to be relevant. ROS leakage from the phagosome into the cytoplasm could provide a mechanism for its effects on Mac-1 –dependent caspase cleavage and PICD. Mac-1 dependent phagocytosis also engages survival pathways, such as the activation of MAPK-ERK. However, ROS-triggered caspase 3 and 8 activation overcomes these survival signals, promoting rapid apoptosis of the phagocytosing neutrophil. A more robust activation of MAPK-ERK also occurs during Mac-1 dependent phagocytosis in the presence of GM-CSF. This results in a decrease of caspase activation and the associated apoptosis, despite an enhanced oxidative burst under these conditions. Thus MAPK-ERK activation generates competing survival pathways that can avert PICD. In summary, Mac-1-mediated phagocytosis in neutrophils integrates pro- and anti-apoptotic signals that eventually favour cell death. These signals are driven by intracellular ROS production, caspase 3 and 8 and MAPK-ERK activation. Additional exogenous stimuli such as TNF and GM-CSF, present at inflammatory sites, exploit these intracellular signals generated by phagocytosis to shift the life-death balance during phagocytosis to either enhanced survival or apoptosis.

β_2-integrin engagement during neutrophil transmigration extends the survival and thus pool of functional neutrophils in tissues, whereas β_2-integrin-dependent phagocytosis of microbes accelerates apoptosis and might be a potent mechanism to eliminate neutrophils that have reached the end of their useful life span. (Mayadas and Cullere, 2005)

Removal of neutrophils by apoptosis is a homeostatic mechanism that prevents damage to healthy tissues that would otherwise occur after necrotic cell lysis. This process is central to the prevention and resolution of inflammation. Neutrophil apoptosis is inhibited in patients with systemic inflammation, systemic infections, severe sepsis and those at risk of multiple organ dysfunction syndrome (MODS). This reduced apoptosis is thought to be due to the activity of circulating factors that

include LPS, lipoteichoic acid and pro-inflammatory cytokines although binding to activated endothelium extends the life-span of neutrophils in contrast to unstimulated endothelium which exacerbates cell death. Soluble fibrinogen can activate neutrophils and delay apoptosis through Mac-1 activation so adhesion is not essential to generate survival signals. The extended life span will contribute to neutrophil mediated tissue damage through release of ROS and proteolytic enzymes. ERK kinase but not p38-MAPK regulates LPS-induced reduction in apoptosis. (Harter et al., 2002)

In adult respiratory distress syndrome (ARDS), IL-2 concentration in the BAL (Bronchoalveolar lavage) correlates with the low level of apoptosis seen. This takes place through a dysregulation of a complex network of intracellular signalling and of organelle function that include an increase in tyrosine phosphorylation and a sustained mitochondrial transmembrane potential. (Melley et al., 2005)

1.21 Cellular regulation of neutrophils

Neutrophils are also regulated by other cells of the immune system particularly T-cells. $CD4^{+}CD25^{+}CD127^{-}$ $FOXP3^{+}$ T-regulatory cells (T-regs) directly inhibit neutrophil function, promoting apoptosis and death when exposed to LPS through binding to surface bound TLR4. This inhibits pro-inflammatory activities, curtailing over exuberant innate immune response and helping to bring about resolution of the inflammatory response. (Lewkowicz et al., 2006) Upon activation, these T-regs can either induce themselves or $CD4^{+}CD25^{-}FOXP3^{-}$ T effectors to differentiate into IL-17A producing cells, Th17, in the presence of TGF-ß and/or IL-6. (Xu et al., 2007) In contrast to the role of T-regs on neutrophils, Th17 cells recruit neutrophils into inflamed tissue further increasing the antimicrobial response *in vitro* and *in vivo*. (Annunziato et al., 2008, Kolls et al., 2008)

1.22 Summary

Neutrophils are powerful antimicrobial effector cells that undergo prolonged development in the BM to equip them with an array of carefully packaged granules. They exist in a resting state in the circulation but can be rapidly primed through chemokine signalling via G-protein coupled receptors. Subsequent activation of protein kinase signalling cascades up-regulate an array of surface receptors which

recognise PAMPs and DAMPs, as well as further chemokine and cytokine signals. Complex interaction with the vascular endothelium leads to recruitment into sites of inflammation within minutes, followed by phagocytosis of invading microbes and/or granular fusion events which culminate in activation of the NADPH oxidase and subsequently other antimicrobial systems. The result in 99% of cases is eradication of the microbial invader and apoptosis leading to pus formation and resolution of infection. However, inappropriate or over activation of neutrophils which release their toxic granular contents can lead to significant tissue collateral damage and organ failure. Next I will review the evidence for neutrophil-induced damage and dysfunction in microbial sepsis, a syndrome with many features akin to acute liver failure (ALF) and liver cirrhosis. Finally the review will focus on the limited literature that supports neutrophil dysfunction in all forms of liver failure on which this thesis is based.

Chapter 2 - Neutrophils and their involvement in disease

2.1 Neutrophil dysfunction, its relationship to sepsis and role in multi-organ dysfunction syndrome (MODS).

Neutrophils are the first line of the host defence against bacterial and fungal infection. Neutrophils have a complex and varied arsenal of antimicrobial weaponry with a high degree of redundancy that is unleashed in a precisely targeted fashion at sites of infection akin to chemical warfare. Neutrophil activation can however, be a double-edged sword, and in severe sepsis inappropriate and excessive release of active protease enzymes and ROS contribute to tissue damage in organs distant to the site of initial infection. This can ultimately lead to organ dysfunction and in the most severe cases MODS with its attendant mortality. (Smith, 1994, Kyriakides et al., 2001) During microbial infection a careful balance must be struck between a sufficient neutrophil response to ensure microbial eradication whilst at the same time minimising collateral tissue damage and ensuring timely resolution of the inflammatory response. (Annane et al., 2005)

MODS is a severe complication of acute or chronic liver failure and severe sepsis with a high morbidity and mortality. Indeed there exist many phenotypic similarities between patients with acute or chronic liver failure and those with severe sepsis, with both conditions being associated with endotoxemia, haemodynamic derangement and abnormal immune responses. (Rajkovic and Williams, 1985) Similar pathophysiological processes are therefore likely to underlie both conditions. Substantial progress has been made over recent decades in the understanding of the role of neutrophils in the immunopathology of severe sepsis but in liver failure this exists at a more rudimentary level. Therefore it is timely to undertake a brief review of the current paradigm of sepsis and the multitude of roles played by neutrophils in that condition.

2.2 Severe Sepsis: definitions and outcomes

It is now well understood that sepsis is associated with a generalised inflammatory reaction in organs remote from the initial insult. This is termed the systemic inflammatory response syndrome (SIRS). In addition it is recognised that non-infectious SIRS can occur in extreme events in patients with trauma, burns, necrotising pancreatitis and liver failure. Severe sepsis is clinically defined by the

presence of a SIRS score along with radiological or laboratory evidence of infection and sepsis-induced tissue hypoperfusion, hypotension or organ dysfunction [Figure 2-1].

SIRS is commonly a feature of hospitalised patients with liver disease often precipitated by infections such as spontaneous bacterial peritonitis (SBP) and pneumonia but equally a sterile SIRS can be seen (e.g. acute liver failure and acute alcoholic hepatitis). In liver disease, differentiation between sepsis and a sterile response clinically is often impossible and indeed the two may co-exist. Patients with SIRS from an infectious or non-infectious aetiology are at risk of progression to MODS; this commonly includes a combination of some or all of the following; haemodynamic instability (septic shock); acute lung injury (ALI); acute kidney injury (AKI) and delirium (or septic encephalopathy). Clinicians who deal with liver failure will be only too familiar with these complications in their patients. Many patients with SIRS and MODS ultimately require specialised management on an intensive care unit (ICU). (Annane et al., 2005) Despite advances in intensive care management mortality from severe sepsis remains shockingly high at 30-70%. (Angus et al., 2001)

Table 2-1 The Systemic Inflammatory Response Syndrome adapted from the 1992 ACCP/SCCM Sepsis definitions

(Bone et al., 1992)

The Systemic Inflammatory Response Syndrome (SIRS)
SIRS = Two or more of the following • Temperature>38°C or <36°C • Heart rate >90 beats per minute • Tachypnoea > 20 breaths per minute or $PaCO_2$ <4.3 kPa • White cell count >12 x 10^9/L, <4 x 10^9/L, or >10% immature (band) forms
Sepsis = SIRS with radiological or laboratory evidence of infection
Severe sepsis = sepsis-induced tissue hypoperfusion, hypotension or organ dysfunction
Septic Shock = sepsis with arterial hypotension despite adequate fluid resuscitation
MODS=failure of 2 or more organs so homeostasis cannot be sustained without support

In severe sepsis excessive activation of neutrophils, due to high circulating levels of cytokines and Toll-like receptor activation by endotoxin (LPS), has been implicated in the pathogenesis of both ALI (Windsor et al., 1993) and AKI. (Thijs and Thijs, 1998, Awad et al., 2009) Animal models of ALI show that neutrophils contribute to lung injury after haemorrhage and infection. (Ayala et al., 2002) Interestingly, neutrophil migration into tissues further alters circulating neutrophils and groups have shown that in murine models of endotoxemia and haemorrhagic shock that lung-derived neutrophils display activation of transcription factors, intracellular kinases and increased expression of inflammatory cytokines; these changes were not observed in circulating neutrophils. (Abraham et al., 2001, Shenkar and Abraham, 1999) In human volunteers similar findings have been observed when endotoxin is instilled at bronchoscopy. (Coldren et al., 2006) Gene expression is dramatically upregulated in neutrophils sequestered to the lungs compared to circulating neutrophils. Of further interest is that neutrophils from inflammatory foci in cystic fibrosis appear to be insensitive to anti-inflammatory signals such as IL-10 or corticosteroids. (Petit-Bertron et al., 2008, Corvol et al., 2003) This suggests that fundamental changes take place in neutrophils after migration to inflammatory foci, a concept termed 'compartmentalisation.' (Cavaillon and Annane, 2006) However, this model of ALI is not the same as systemic neutrophil activation as seen in SIRS or severe sepsis. The large number of primed circulating neutrophils in sepsis marks them out as prime suspects in mediating much of the damaging aspect of a robust immune response.

2.3 Initiation of SIRS

The response to bacterial and fungal infection is initiated following the detection of 'danger signals' by sentinel immune cells such as tissue macrophages and dendritic cells. Two groups of molecules are critical in this regard, the highly conserved microbial molecules called pathogen-associated molecular patterns [PAMP] (Medzhitov, 2001) and disease-associated molecular patterns [DAMP] (Chen et al., 2007) released as a consequence of tissue damage or apoptosis during bacterial invasion. Once PAMPs and DAMPs ligate with their respective PRRs on these sentinel cells they release pro-inflammatory mediators (e.g. IL-1β and TNF), neutrophil chemokines and lipid mediators. The resultant activation of local vascular endothelium leads to neutrophil recruitment as outlined in chapter 1. The generation

of DAMPs in situations of sterile inflammation, such as acute and chronic liver disease can generate a SIRS response including cytokine generation and immunomodulation. High mobility group box-1 (HMGB-1), a nuclear factor released by apoptotic cells, is an important DAMP acting as a late mediator of sepsis. (Wang et al., 1999) Levels of HMGB-1 are increased in acute liver failure (ALF) and are released from the necrotic liver; (Craig et al., 2011) through subsequent TLR4 activation this can generate a similar immunophenotype to sepsis. (Andersson and Tracey, 2011)

2.4 Compensatory Anti-Inflammatory Response Syndrome

The hallmark of severe sepsis and SIRS is the generation of large quantities of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, from immune cells in a so-called 'cytokine storm'. (Cavaillon and Annane, 2006) However, of equal importance is the concomitant generation of a compensatory anti-inflammatory response syndrome (CARS), which can be considered the counterbalance to SIRS preventing an overzealous pro-inflammatory response. This ultimately acts as the 'off switch' to SIRS following successful resolution of pathogenic invasion. If CARS becomes excessive or protracted it may become pathological preventing clearance of infection and resolution of organ failure or predisposing to secondary infection.

CARS is characterised by the production of anti-inflammatory cytokines such as soluble tumour necrosis factor receptor (sTNFR), IL-10, IL-1 receptor antagonist (IL-1Ra) and transforming growth factor-β (TGF-β). CARS occurs alongside the initial cytokinaemia of SIRS, being an adaptive response to facilitate resolution of the inflammatory response. (Munford and Pugin, 2001) However, high levels of anti-inflammatory mediators in the blood stream correlate strongly with down-regulation of the immune response and a poor outcome from sepsis. (Bone et al., 1997) It is well established that the plasma of patients with sepsis has the capacity to inhibit leukocyte functions and can therefore be considered an immunosuppressive milieu. (Cavaillon, 2002) Further evidence for an altered immune status in sepsis and SIRS comes from the high incidence of nosocomial infection in such patients, anergy to skin test antigen and reduced neutrophil adherence and chemotaxis (Meakins et al., 1977, Christou, 1985) Lymphocyte abnormalities are observed in response to IL-10 with reduced responsiveness and reduced numbers of both circulating and tissue lymphocytes.

(Friedman et al., 1997) The action of these two opposing and rapidly changing signalling events in patients with severe sepsis gives rise to the concept of a balance between SIRS and CARS. A predominance of either state referred to as 'immune dissonance' can lead to a poor outcome from sepsis. The immune system is therefore constantly adapting to bring about balance. Eventually there is a swing towards CARS bringing about resolution of infection. The immunosuppressive effects of CARS persist following resolution from the initial insult and the clinical consequences of this are a high incidence of nosocomial infections in intensive care populations. (Volk et al., 2000)

2.5 Neutrophils in severe sepsis

Neutrophils certainly possess the mechanics to cause significant cellular damage with production of ROS and potent serine proteases. Soluble mediators such as PMA have been shown to induce granule fusion with the plasma membrane and subsequent extracellular release of these elements. (Borregaard et al., 1983) This is clearly desirable to localise infection and when successful, leads to abscess formation with containment of infection. However, the systemic neutrophil activation seen in sepsis means that these actions can take place distal to the site of infection resulting in tissue damage and organ failure. Post mortem analysis of patients who die of MODS provides support for this theory with accumulation of neutrophils in renal blood vessels and large-scale tissue infiltration in ALI has been observed. (Gao et al., 2002) Two commonly recognised phenomena need to be explained for the central role of neutrophils in this cellular damage to holdfast; those are the apparent discordance between the degree of tissue damage observed and organ function, and the augmented SIRS response in neutropenic sepsis. Cell hibernation may explain the apparent lack of concordance between tissue damage and organ function, a process whereby oxidative cellular stress leads to impaired mitochondrial function. (Gabay and Kushner, 1999) Neutrophils can produce massive quantities of ROS potentially contributing significantly towards the mitochondrial oxidative stress postulated to mediate 'cell hibernation'. A small number of primed neutrophils rapidly recruited to tissues with subsequent activation by cytokines and chemokines may be sufficient to cause significant tissue destruction and oxidative damage resulting in organ failure. Clearly neutrophils operate alongside other immune cells but their large numbers,

rapid recruitment and toxic potential point to them having a critical role in the organ failure of severe sepsis.

2.6 The generation of peripheral neutrophilia

One of the early features of infection is an increase in neutrophil numbers and the proportion of immature 'band' forms, the so called 'left-shift'. Neutrophilia is a defining characteristic of the SIRS response and the peripheral neutrophil count can triple within 4 hours. Peripheral neutrophilia results from increased granulopoiesis, increased mobilisation of neutrophils from the BM, demargination (neutrophils entering the peripheral circulation from areas of intravascular 'marginated' cell pools such as the lung), and reduced removal through apoptosis.

GM-CSF and other circulating chemokines such as IL-6 and IL-8 appear to play critical roles in stress granulopoiesis. (Zhan et al., 1998, Kopf et al., 1994, Starckx et al., 2002) However, the BM possesses large stores of retained neutrophils with egress being regulated by CXC-chemokine receptor CXCR2 and CXCR4 expression on the cells. CXCR4 activation retains neutrophils within the BM whereas CXCR2 facilitates egress. The dominant production of the CXCR4 ligand SDF-1 from osteoclasts results in neutrophil retention. G-CSF and GM-CSF mobilises neutrophils from the BM during sepsis by inhibiting osteoclast production of SDF-1 and increasing the production of the CXCR2 ligands keratinocyte derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2). (Semerad et al., 2002) Other ligands involved include corticosteroids, endotoxins, complement-derived leukocyte-mobilising factor, C5a chemoattractant, TNFα, and androgens. Mobilisation of BM reserves may result in the release of immature or early myeloid forms known as band forms. Toxic neutrophils are observed in the peripheral circulation of patients with sepsis and possess various morphological abnormalities. Initially changes were assumed to be due to effects of endotoxemia, however, it is now understood that these changes are acquired during maturation under extreme conditions that intensely stimulate neutrophil production and shorten maturation time. These changes can be induced by G-CSF. Most of the changes reflect asynchrony of maturation between the nucleus and the cytoplasm resulting in the persistence of the protein synthetic machinery such as RNA, ribosomes and rough endoplasmic reticulum (RER). This leads to the presence of phenomena such as Dohle bodies (whorls of RER), basophilic

cytoplasm (polyribosomes and RER), toxic granulation (eosinophilic primary granules usually absent), nuclear immaturity (lighter and less closely clumped chromatin) and cytoplasmic vacuolisation (frothy cytoplasm due to lysosome degranulation). The presence of toxic neutrophils and cytoplasmic vacuoles can reflect neutrophil autolysis in response to endotoxemia but can develop as a storage artefact after 4 hours of removal from the body.

Sepsis and the acute phase response lead to the demargination of neutrophils adherent to the endothelium in the lungs and other organs in response to cytokines such as IL-6. (Suwa et al., 2000) Adrenaline and corticosteroids that may be used to treat patients with sepsis in the ICU can also cause demargination, (Brenner et al., 1998).

Neutrophil apoptosis is characteristically reduced in sepsis and SIRS with delay induced by G-CSF, GM-CSF, LPS, LTA, and TNFα. Neutrophil life span is additionally increased when bound to vascular endothelium that is activated by pro-inflammatory mediators whereas binding to unstimulated endothelium induces apoptosis. (Ginis and Faller, 1997) A reduced release of Cathepsin-D by neutrophils in sepsis, an activator of caspase 8, may be a further mechanism for reduced neutrophil apoptosis. Apoptosis is promoted by the effects of IL-10 thus readdressing the balance once CARS predominates leading to recovery from inflammation. (Keel et al., 1997) Interestingly however, clinical trials using G-CSF and GM-CSF to increase the number and maturity of neutrophils have had disappointing results with no improvement in mortality from sepsis reported. (Root et al., 2003, Nelson et al., 1998) However, mortality has been shown to improve in patients with chemotherapy-induced neutropenia. (Maher et al., 1994)

Neutrophilia whilst a marker of infection and component of the SIRS score does not correlate with severity of organ failure. There is recognition in the SIRS score that neutropenia can be a marker of severe sepsis (Figure 7), often in elderly and neonates, although it is unclear if this is an inadequate response or recruitment of neutrophils to tissues with subsequent inappropriate activation.

2.7 Neutrophil surface receptor expression

Neutrophils from patients with sepsis display a primed phenotype with respect to adhesion molecule expression, shedding L-selectin [CD62L] (Gustot et al., 2009) and

up-regulating surface expression of CD11b (MAC-1) (Arvaniti et al., 2010) and CD49d (VLA-4) [Table 2-2]. (Ibbotson et al., 2001) Shedding L-selectin, which mediates the brief 'on-off' interactions between neutrophils and the endothelium reduces neutrophil margination, whereas increased CD11b and VLA-4 levels coupled with increased CD11b affinity, (Schleiffenbaum et al., 1989) favours firm adhesion to activated endothelium at the site of infection. The source of this receptor upregulation is through granular fusion with the plasma lemma. Neutrophils from patients with sepsis show enhanced adhesion to activated endothelial membranes although inhibition of CD11b and β2-integrins does not abolish this suggesting a role for other cell adhesion molecules such as VLA-4. One critical question is how is neutrophil recruitment facilitated at sites distant from the initial infection? Clearly there is a role for circulating PAMPs, DAMPs and inflammatory mediators. For example, TNFα is a key mediator of septic encephalopathy causing alterations in blood-brain barrier function and upregulation of adhesion molecules that mediate neutrophil recruitment to tissues following an inflammatory stimulus. (Alexander et al., 2008)

Levels of the high affinity gamma-globulin receptor CD64 are increased in neutrophils from septic patients, a receptor not normally expressed on resting neutrophils, correlating with levels of the chemokine IL-8, severity of sepsis and mortality. (Qureshi et al., 2001, Livaditi et al., 2006) Recently CD64 has been shown to be a robust marker for survival when used with a combination of other septic biomarkers. (Gibot et al., 2012) Work from our group has questioned whether CD64 upregulation is a phenomenon unique to sepsis, as a similar upregulation is observed in ALF in a sterile inflammatory setting resulting from hepatocellular necrosis. (Abeles et al., 2012) Interestingly, neutrophils with enhanced expression of CD64 show augmented respiratory burst capability providing weight to the argument that neutrophils in sepsis are mediators of bystander tissue damage.

TLR2 but not TLR4 is increased on the surface of neutrophils by G-CSF and GM-CSF. (Kurt-Jones et al., 2002) Agonists of either receptor initiate a respiratory burst, release IL-8, shed L-selectin and increase CD11b expression. The expression of CD14, the LPS co-stimulatory molecule normally expressed at low levels on neutrophils (predominantly being a monocyte/macrophage receptor) is increased in neutrophils from patients with sepsis.

A further PRR triggering receptor on myeloid cells (TREM-1) has shown increased expression on neutrophils in sepsis but appears confined to acute inflammatory reactions. Recognition of bacteria by complement, TREM-1 and TLR are likely to be more important in the early stages of infection as IgG levels will be low prior to activation of B-cell and the adaptive immune system. The redundancy in the system means that targeting individual cytokines is unlikely to be successful and indeed anti-TNFα antibody (Fisher et al., 1996) has proven unsuccessful in sepsis.

IL-8 is a ligand for the high affinity chemokine receptor CXCR2 and reduced expression of this receptor is seen on the surface of neutrophils from septic patients associated with a markedly suppressed response to IL-8. (Cummings et al., 1999) However, CXCR1 levels are unchanged and there are contradictory reports that neutrophil responsiveness is impaired or normal. Mouse models where blockade of CXCR1 and CXCR2 signalling is achieved can prevent MODS and disseminated intravascular coagulation. (Kaneider et al., 2005) Further evidence for the importance of the action of IL-8 comes from patients with ALI where neutrophils undergo a large-scale migration into the lungs. Moreover, concentrations of IL-8 in bronchoalveolar lavage (BAL) fluid correlate with mortality. (Panasiuk et al., 2005) In the lumen of the pulmonary capillaries there is enforced neutrophil contact with the vessel wall unlike in other organs where adhesion and migration occur in the post-capillary venules. Binding of neutrophils to the endothelium additionally prolongs neutrophil life span by reduction in apoptosis. IL-8 therefore appears to be a critical chemokine in neutrophil priming, adhesion and activation in sepsis. A further chemokine receptor showing upregulation on neutrophils from sepsis is the fMLP receptor with an enhanced response to fMLP being reported as a marker of neutrophil priming. (Tschaikowsky et al., 1993)

Activation of TLRs and chemokine receptors leads to enhanced expression of the intracellular transcription gene, NFκB as a consequence of neutrophil priming in response to ligation of PRR during neutrophil priming. Indeed, neutrophils from patients with sepsis demonstrate upregulation of NFκB and reduced activation of NFκB is associated with improved survival. (Nakamori et al., 2003) NFκB is the main transcription factor required for the expression of the genes coding for inflammatory molecules. For a summary of the changes to neutrophil surface markers in sepsis see Table 2-2.

Table 2-2 Change in neutrophil cell-surface receptor expression in sepsis

Neutrophil phenotype and function in patients with sepsis	**Change with respect to normal neutrophils**
Adhesion molecules	
L-selectin β2 integrins CD11b β1 integrins VLA-4 (CD49d)	Down regulated Upregulated and enhanced affinity Upregulated
Chemokine receptors	
CXCR1 CXCR2	Unchanged Down regulated
Fcγ Receptors	
CD16 CD32 CD64	No change No change No change
Pathogen recognition receptors	
TLR-2 TLR-4 TREM-1	Upregulated No change Upregulated

2.8 Neutrophil functional status in sepsis

There is a lack of clarity regarding the functional activities of neutrophils in sepsis and with contradictory reports in the literature. The veritable potpourri of findings may be explained by the literature being peppered by the use of a variety of models, assays and conditions under which these studies have been performed. Another critical point in the understanding of the immunopathology that occurs in sepsis is that the changes are highly dynamic with an evolving picture changing over time depending on the predominance of a SIRS or CARS response and the specific microbial challenge.

One of the key and best understood of the neutrophil abilities is their homing to areas of infection and this is perhaps most clear in relation to neutrophil function. Not surprisingly therefore, enhanced neutrophil adhesion has been recognised in sepsis for some time with early studies demonstrating enhanced adhesion to petri

dishes and nylon threads in response to LPS stimulation. This is achieved *in vivo* by a change in the phenotype of the cell surface expression ('priming') through shedding of L-selectin and enhancing the number and affinity of Mac-1 and non-integrin binding sites results in enhanced binding at sites of infection. (Dahinden et al., 1983, Parent and Eichacker, 1999) The 'primed' phenotype should inhibit recruitment to uninvolved tissue beds. However, in severe sepsis there is perturbation of this homeostatic mechanism with systemic neutrophil 'priming' being associated with disseminated leukocyte recruitment to organ beds distant to the initial source of infection. It is therefore interesting to note that L-selectin shedding and integrin dysfunction render leukocyte adhesion increasingly susceptible to the effects of sheer stress and alternative adhesion molecules. Through this mechanism 'primed' neutrophils are inhibited from recruitment to normally perfused sites with intact endothelium but can be recruited to sites with compromised perfusion or endothelial injury. (Ploppa et al., 2010) Interestingly, some studies suggest that in severe sepsis recruitment to local sites of infection is actually impaired. (Ahmed et al., 1999)

Neutrophil migration is reportedly defective in skin blisters in patients with sepsis (Merli et al., 2010). Neutrophils from patients with sepsis have a reduced expression of CXCR2 but no reduction in CXCR1 with a chemotactic responsiveness to IL-8 that may be impaired (Chishti et al., 2004) or normal. (Cummings et al., 1999) Migration of isolated neutrophils in response to LT-B4 is impeded in sepsis (Tavares-Murta et al., 2002) but it is unclear if migration in response to fMLP is altered in sepsis as conflicting reports have been published. (Tavares-Murta et al., 2002, Cummings et al., 1999)

The majority of authors report a reduced neutrophil phagocytic capacity in sepsis but this is controversial with other studies reporting both increased and reduced levels of phagocytosis. (Zhang et al., 1994, Simms et al., 1991) This is a paradoxical finding to what one would expect as neutrophil 'priming' induces surface upregulation of opsonin receptors such as CD11b (complement receptor) and high affinity IgG receptor expression. Phagocytosis by immature neutrophils in septic patients has been reported to be lower than mature neutrophils a finding that may be important when there is a high proportion of band forms present in the peripheral blood smear. On the other hand, it has been postulated that older neutrophils with the inhibition of apoptosis may lead to neutrophil exhaustion.

Equally, conflicting reports regarding the generation of ROS by neutrophils in sepsis are found in the literature with authors concluding that an up- or down-regulation is observed. (Wenisch et al., 1999, Martins et al., 2003) Kaufmann et al provide some explanation by demonstrating that neutrophil ROS generation in sepsis is stimuli dependent; hydrogen peroxide generation being reduced in response to Zymosan, unchanged in response to PMA and enhanced in response to TNFα and fMLP. (Kaufmann et al., 2006) The timing after an insult where neutrophil function is evaluated may be important with priming and activation being only observed for 24 hours after an insult in burns patients. Subsets of circulating neutrophils have been defined with the presence of both a 'primed' and normal subgroup. (Bass et al., 1986)

2.9 Mechanisms for neutrophil hypo-responsiveness in sepsis

Endotoxemia activation through TLRs and their downstream effects are critically important events in the activation of immune cells particularly in their response to gram-negative sepsis. *Ex vivo* cytokine production of IL-1β, IL-1R and IL-8 is reduced after exposure to LPS in neutrophils obtained from septic patients compared to healthy controls. (McCall et al., 1993, Marie et al., 1998) This has been further confirmed after the intravenous administration of LPS in healthy human volunteers whose neutrophils displayed a reduced capacity to produce chemokines upon *in vitro* stimulation. (Schultz et al., 2000) This neutrophil hyporesponsiveness has been compared to the 'endotoxin-tolerance" described in monocytes where prior exposure to minute quantities of endotoxin *in vitro* renders them refractory to re-challenge with the same agonist. (West and Heagy, 2002) Circulating monocytes isolated from patients with sepsis typically display a reduced production of TNFα and unaltered or enhanced production of IL-10. (Cavaillon and Adib-Conquy, 2006) Similar findings have been shown in patients with a variety of sterile SIRS responses such as ALF and following LPS administration in healthy controls. (Antoniades et al., 2006, Granowitz et al., 1993) The precise mechanism leading to endotoxin tolerance is unclear but involves reduction in surface expression of TLR4 and further down-stream receptor-effector uncoupling. (West and Heagy, 2002) Interestingly however, the induction of endotoxin tolerance in mice enhances their resistance to fungal and

bacterial infections. (Rayhane et al., 2000, Lehner et al., 2001) A similar phenomenon of neutrophil hypo-reactivity has been reported in sepsis and SIRS.

A more recent study by Parker et al. shows that prolonged exposure of *ex vivo* neutrophils from healthy controls to LPS results in induction of tolerance in intracellular signaling pathways (including p38-MAPK) and respiratory burst. Tolerized neutrophils retain the ability to delay apoptosis in response to GM-CSF, show continued generation of IL-8 and reduced surface expression of TLR4 (Parker et al., 2005) suggesting that the neutrophils are reprogramed but maintain a pro-inflammatory phenotype.

Serum from septic patients has potent inhibitory effects on circulating neutrophils and monocytes and is a so-called 'inhibitory milieu'. (Constantian and Cohen, 1978, Prins et al., 1995) Inhibitory factors can be detected in plasma from healthy volunteers after LPS exposure and can be removed by plasma filtration and adsorption. (Ronco et al., 2002) Such factors include soluble CD14, LPS-binding protein, high-density lipoprotein (HDL), soluble myeloid differentiation protein-2 (MD-2) as well as the anti-inflammatory cytokines IL-10 and TGF-β. (Adib-Conquy and Cavaillon, 2009) IL-10 release can be favored by the action of catecholamines that are known to contribute to the altered responsiveness of circulating leukocytes. β-Blockers allow a partial restoration of the responsiveness of blood leukocytes to LPS in terms of TNF production. (Asehnoune et al., 2006) Indeed, β-blockers reduce the mortality due to spontaneous bacterial infection post stroke (Prass et al., 2003) as opposed to norepinephrine-treated neutrophils which decrease the resistance of mice to infection. (Tsuda et al., 2008)

One of the most fascinating observations that support the necessity for a balance in the production of pro- and anti-inflammatory responses is the observation that neutrophils can be rendered either hyper or hypo-responsive dependant on the severity of the initial septic/inflammatory insult. Severe insults such as acute pancreatitis lead to an increased susceptibility to spontaneous bacterial infection leading to increased mortality. (Suzuki et al., 2006) Moreover modest stimulation induces resistance to a secondary infection (pancreatitis and burns) in caecal-ligation and puncture animal models. (Echtenacher and Mannel, 2002) Low grade priming stimuli such as prior LPS exposure dramatically reduces neutrophil activation and adhesion is associated with an elevated circulating neutrophil count, low degree of

micro-vascular plugging, and survival after a normally lethal dose of LPS. (Barroso-Aranda et al., 1991) Some authors have contradicted these findings suggesting that responses to polymicrobial and monomicrobial infection may differ and the timing of the insult and secondary infection may be relevant. Neutralisation of IL-10 and the CCL chemokine monocyte chemoattractant protein-1 (MCP-1) reduce the deleterious effect, suggesting they are both serum factors that mediate this effect. (Reich et al., 1997, Tsuda et al., 2004)

2.10 Summary of neutrophil dysfunction in sepsis

In severe sepsis neutrophils turn from hero to villain with generalised neutrophil priming and activation leading to recruitment at distant sites from the initial infection as a result of endothelial injury. Once recruitment occurs neutrophils release toxic proteases and ROS with subsequent cell and mitochondrial toxicity resulting in organ failure. This is reversible over time but unchecked, neutrophil damage leads to MODS with its associated poor prognosis. The complex interplay between pro- and anti-inflammatory signals being generated requires a balanced, dynamic and proportionate cytokine and chemokine response in order for successful resolution from infection to occur. Post infection and after prolonged exposure to pro-inflammatory stimuli there is immune hypo-responsiveness contributing to recurrent infection. An understanding of this process may allow manipulation to prevent both an over-exuberant response and the development of secondary infection in the immunosuppressive phase. The immune dysfunction observed in sepsis and in liver failure share many similarities and indeed are likely to have similar mechanisms that underpin them. In addition cirrhosis adds a further dimension with the development of chronic immune dysfunction having no “off” switch.

Chapter 3: Neutrophils in acute and chronic liver failure

3.1 The burden of bacterial infection in acute and chronic liver failure

Both acute and chronic liver failure are associated with an increased incidence of bacterial and fungal infection leading to hospitalisation and the development of potentially life-threatening complications.

Sepsis can lead to worsening liver function and precipitate complications, including variceal bleeding, hepatic encephalopathy (HE), AKI and MODS, contributing to high mortality. Cirrhosis represents a significant health burden in the United Kingdom (UK) with the incidence and prevalence of the condition increasing exponentially; it is now the fifth largest cause of death. (Blachier et al., 2013, BASL and BSG, 2009) The increasing burden of liver disease poses a major challenge in managing these patients particularly with respect to utilisation of ICU beds and requirement for Liver Transplantation (LT). With poor outcomes following sepsis and its associated complications, and increasing waiting list mortality for LT, there is an urgent need for novel approaches to reducing the rate of infection in cirrhosis.

ALF is a rare but frequently catastrophic consequence of acute severe hepatic injury arising from a wide variety of insults. It is similarly associated with a high incidence of early and late bacterial and fungal infection resulting in clinical deterioration and MODS. ALF patients who fulfil poor prognostic criteria are at high risk of death without LT, and complicating infection prevents listing for LT. Infection is frequently seen to complicate the post-operative course following LT in ALF with associated mortality contributing to over 25% of deaths (Rolando et al., 1990). LT at present represents the only curative therapy in end-stage chronic liver disease (CLD); however, with a shortage of donors for those eligible for LT up to 20% die while on the waiting list.

3.2 The incidence of infection in cirrhosis

Infection and associated endotoxemia is present on admission or complicates 32-34% of hospitalised patients with cirrhosis. (Fernandez et al., 2002, Borzio et al., 2001, Navasa et al., 1999) Those with the most severe liver failure are the most susceptible to infection with correlation seen between Child-Pugh score (CPS) and rates of infection in cirrhosis. (Borzio et al., 2001) CPS is a prognostic measure of liver disease severity with recognised grades A, B and C each referring to worsening 2-year

survival as you progress from A through to C. (Pugh et al., 1973) Patients with more advanced disease are additionally more susceptible to developing organ dysfunction suggesting that immune dysfunction could be related either to liver failure or portal hypertension. The in-hospital mortality from infections in cirrhosis is 15-31% and in septic shock can be as high as 70%. (Plessier et al., 2003) Other risk factors for infection in cirrhosis include variceal bleeding, low ascites protein concentration (<10-15mg/L), previous episodes of spontaneous bacterial peritonitis (SBP), HE and invasive procedures.

Up to 35% of patients with ALF develop bacterial and fungal infection with a median time to bacteraemia of 10-days; independent predictors of infection include high SIRS score and the presence of HE. (Karvellas et al., 2009, Rolando et al., 1991)

3.3 Aetiology of infection in liver failure

Common infections include SBP, pneumonia, procedure-related bacteraemia, cellulitis and spontaneous bacteraemia. (Fernandez et al., 2002) Cirrhotic patients are more at risk of infection from atypical organisms such as tuberculosis and invasive fungal infections. 39% of infections are nosocomial with a high rate of bacteraemia from invasive procedures such as central venous cannulation, paracentesis and urinary catheterisation. Infections are culture positive in 50-70% of cases. In community infection the main causative agents are gram-negative cocci (GNC), such as *E.coli,* in 60%, with gram-positive cocci (GPC) found in 30-35% and a mixed culture in the remaining 5-10%. In nosocomial infection the trend is reversed with 60% GPC and 30-35% GNC, as a result of prior antibiotic exposure and invasive procedures. Other common organisms include *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus spp*. In SBP there is evidence that less virulent strains of *E.coli* are responsible for infection with worsening liver failure (Bert et al., 2008) and fungal infections are more common e.g. *Candida spp*. In ALF bacteriologically proven infection occurs in up to 80% of patients, 70% of bacterial isolates are GPC and fungal infection develops in up to 32%. (Rolando et al., 1996)

3.4 Immune dysfunction in chronic liver failure and predisposing factors

Cirrhosis represents a state of functional immune paresis and the term cirrhosis associated immune dysfunction syndrome (CAIDS) has been coined. (Bonnel et al., 2011) CAIDS comprises a number of wide-ranging immune defects in innate and adaptive immune responses along with up-regulation of pro-inflammatory cytokine production. The immune dysfunction shares many similarities to that seen in patients with sepsis suggesting a similar aetiopathogenesis. (Wasmuth et al., 2005)

A number of factors predispose to infection in cirrhosis including advancing severity of liver disease (Yoshida et al., 1993), variceal bleeding, (Bernard et al., 1999, Borzio et al., 2001) low ascites protein levels (Llach et al., 1992, Such et al., 1988) and prior episodes of SBP. (Gines et al., 1990) The underlying immune defect is complex and multi-faceted and contributory factors include protein malnutrition, alcohol consumption, and BM suppression. This occurs in association with multiple defects principally of the innate but also subtle defects of the adaptive immune system. (Rajkovic and Williams, 1985, Bolognesi et al., 1994, Shawcross et al., 2008b) In addition, drug therapies such as corticosteroids, immunosuppressant and antiviral therapies further exacerbate the situation in autoimmune and viral aetiologies.

3.5 Disease paradigm of innate immune dysfunction, sepsis, and organ failure in cirrhosis

Portal hypertension is the principle direct complication of cirrhosis being defined as elevation of the hepatic venous pressure gradient (HVPG) above 5mmHg, with clinically significant portal hypertension seen at a HVPG $\geq$ 10mmHg. (Groszmann et al., 2005) An important consequence of portal hypertension is intestinal bacterial overgrowth, enhanced bacterial translocation from the gut, and porto-systemic shunting. (Morencos et al., 1995, Chang et al., 1998)

Bacterial translocation is the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes. (Wiest and Garcia-Tsao, 2005) In cirrhosis slowed intestinal transit with consequent small bowel bacterial overgrowth and reduced mucosal barrier function is found; this is exacerbated with worsening

portal hypertension. (Chesta et al., 1993) Bacterial translocation leads to an increase in the delivery of bacterial derived molecules into the portal vein. Normally these would be removed and neutralised by the reticulo-endothelial (RE) system of Kupffer cells and LSEC present in the liver sinusoids. However, porto-systemic shunting, whereby blood from the portal circulation bypasses the liver, reduced RE function, and direct draining of the RE into the systemic circulation culminates in increased levels of circulating endotoxin and other PAMP molecules. (Riordan et al., 2003, Llovet et al., 1998, Chan et al., 1997) This systemic 'spill-over' of bacterial products leads to circulating monocyte dysfunction and neutrophil priming with subsequent generation of ROS, pro-inflammatory cytokines, and nitric oxide (NO) resulting from endotoxemia and leading to endothelial dysfunction. This further exacerbates the breakdown in intestinal barrier function. (Wiest and Garcia-Tsao, 2005) In addition to portal hypertension, liver cirrhosis can have direct effects on innate immune function, through reduced production of opsonic proteins and neutrophil dysfunction resulting from reduced clearance of ammonia and sodium imbalance. (Runyon et al., 1985) The resulting systemic immune dysfunction facilitates bacterial access to the blood stream and peritoneal cavity ultimately predisposing to infections such as SBP and disrupting the immune response to infection. (Runyon et al., 1994)

Along with immune dysfunction, cirrhosis results in progressive haemodynamic alterations following systemic and splanchnic vasodilatation due to endothelial dysfunction and as a consequence of increased circulating vasoactive substances, leading to effective arterial hypovolemia. (Blendis and Wong, 2001) In order to counterbalance vasodilatation there is upregulation in the production of a number of vasoconstrictors including activation of the renin-angiotensin aldosterone system (RAAS) and release of anti-diuretic hormone (ADH) complimenting increased output from the sympathetic nervous system.

In advanced cirrhosis, portal hypertension and ineffective sodium handling lead to increased peritoneal fluid production (ascites) beyond the ability for lymphatic resorption with subsequent accumulation. Ascitic fluid accumulation secondary to portal hypertension results in a low protein transudate with consequent low levels of opsonins such as immunoglobulin and complement proteins. This reduced opsonic capacity combined with bacterial translocation and neutrophil dysfunction can lead to the development of SBP, one of the most common infections in decompensated cirrhosis.

Infections such as SBP are associated with an exaggerated pro-inflammatory response or 'cytokine storm' leading to further perturbation of endothelial function and deterioration of the precarious haemodynamic status that exists in advanced cirrhosis. Hypovolaemia is further amplified by the presence of cirrhotic cardiomyopathy (defined as a systolic incompetence under stress, diastolic dysfunction and electrophysiological abnormalities) and relative adrenal insufficiency. Infection can consequentially exaggerate the effects of pre-existing hypovolaemia leading to hypotension, with AKI being the commonest extra-hepatic organ dysfunction. In severe sepsis, hypovolaemia results in inadequate tissue perfusion in a situation termed shock which if left uncorrected, leads to MODS requiring support in ICU. The disease paradigm of immune dysfunction in cirrhosis is summarised in figure 3-1.

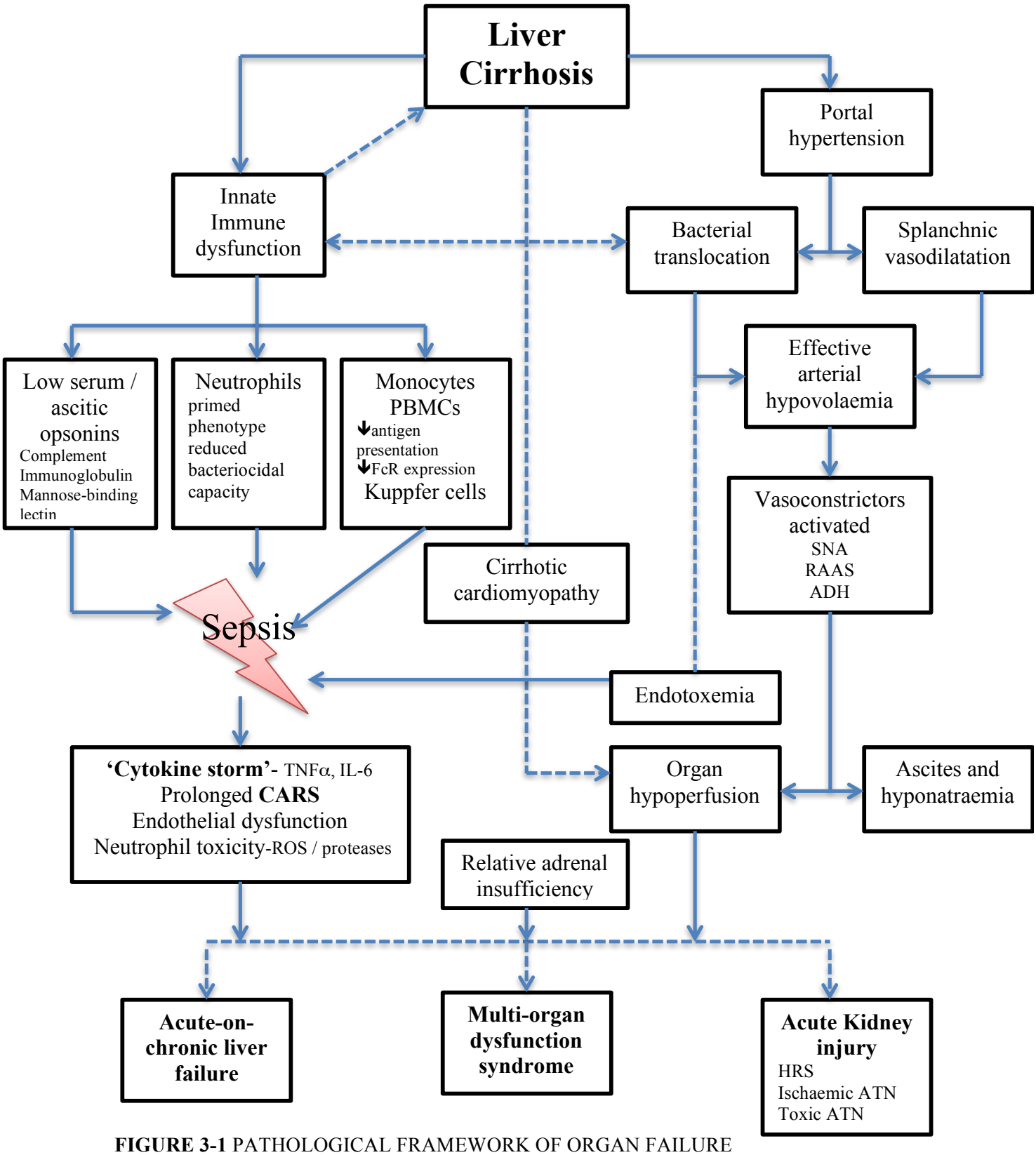


FIGURE 3-1 PATHOLOGICAL FRAMEWORK OF ORGAN FAILURE IN SEPSIS

3.6 Acute-on-chronic liver failure

A further consequence of sepsis is deteriorating hepatic function resulting in a poorly characterised syndrome referred to as acute-on-chronic liver failure' (AoCLF). AoCLF is associated with poor short-term prognosis. (Jalan et al., 2012a) AoCLF is defined as the development of deteriorating hepatic function and extra-hepatic organ failure in patients with cirrhosis as a consequence of an acute event such as SBP and variceal bleeding. (Sen et al., 2002) Diagnostic criteria have recently been refined with grades of severity defined based on a cohort of 1343 patients from 29 European liver units. (Moreau et al., 2013) AoCLF can in itself lead to further events through worsening portal hypertension and synthetic liver dysfunction (including coagulopathy) leading to complications such as variceal bleeding and HE. AoCLF may be directly related to the pro-inflammatory response with TNFα inducing a pro-apoptotic signal within the hepatocytes due to down regulation in translation of NF-κB-dependent anti-apoptotic messenger ribonucleic acids (mRNA) into proteins as a result of endoplasmic reticulum (ER) stress. Moreover, livers from LPS-challenged rats with cirrhosis exhibit an endothelin-1-mediated neutrophil infiltrate and hepatocyte necrosis. (Urbanowicz et al., 2004) At present the underlying mechanism(s) leading to AoCLF are poorly understood but neutrophils may play a key role as discussed below.

Evidence for a role of neutrophils in organ damage is seen in acute alcoholic hepatitis (AAH), a condition where neutrophil recruitment to the liver leads to severe necroinflammation (Bautista, 2002, Jaeschke, 2002) This condition is associated with elevated levels of IL-6, IL-8, IL-10, TNFα and sTNFαR1 (Stadlbauer et al., 2006), which corresponds with severe neutrophil dysfunction and a high incidence of HE and renal failure (Sen et al., 2004). It seems likely that priming of circulating neutrophils and the cytokinaemia contribute to the organ failure observed in this condition.

3.6.1 Circulatory failure

Cirrhosis is characterised by a hyperdynamic circulation, with high cardiac output, low mean arterial pressure and reduced systemic vascular resistance. This situation is further exacerbated by infection due to associated endothelial dysfunction demonstrated by hypo-reactivity to α-adrenoceptor agonists. (Ruiz-del-Arbol et al.,

2003) Other important factors are likely to include cardiac dysfunction, either due to cirrhotic cardiomyopathy or sepsis-induced left-ventricular dysfunction, (Parker et al., 1984) and relative adrenal insufficiency. (Tsai et al., 2006)

3.6.2 Acute kidney injury (AKI)

AKI occurs in 30-33% of patients with cirrhosis who have SBP and in 27% of those with sepsis unrelated to SBP. (Follo et al., 1994) The development of AKI is associated with high levels of pro-inflammatory cytokines (TNFα and IL-6), NO metabolite levels and arterial under-filling. (Navasa et al., 1998) The cause of AKI in severe sepsis is multifactorial and can include pre-renal failure due to circulatory dysfunction, type-1 hepatorenal syndrome (HRS) [unresponsive to filling] and acute tubular necrosis.

3.6.3 Respiratory failure

Neutrophils are important in the aetiology of ALI in severe sepsis without cirrhosis. Indeed, there is a high incidence of acute respiratory distress syndrome (ARDS) in patients with cirrhosis. (Doyle et al., 1995) In rodent models of cirrhosis there are increased numbers of neutrophils marginating to the pulmonary circulation, susceptibility to LPS-induced lung oedema, and death. (Chang and Ohara, 1994) Patients with cirrhosis requiring mechanical ventilation have mortality rates above 50% in studies and indeed the requirement for mechanical ventilation on day-1 in patients with cirrhosis along with the MELD score is predictive of early mortality with an OR 5.7. (Bahirwani et al., 2013, Shawcross and Wendon, 2009)

3.6.4 Neurological failure and hepatic encephalopathy

Neutrophils, along with ammonia and the SIRS response, have been directly implicated in the pathogenesis of HE. (Shawcross et al., 2010) Enhanced endothelial cell interaction in the cerebral circulation may lead to recruitment of activated neutrophils resulting in endothelial disruption and oxidative stress. In sepsis acute neurological deterioration is common and termed septic encephalopathy. (Sprung et al., 1990) Acute neurological deterioration is seen in 21-33% of patients with cirrhosis

and 60-68% in those with septic shock. (Sort et al., 1999) There appears to be a synergism between SIRS and hyperammonemia in the development of neuropsychiatric impairment in cirrhosis. Provoked hyperammonemia induces neurological impairment in patients with cirrhosis and SIRS, which is associated with high levels of TNFα, IL-1β, IL-6 and NO metabolites; this effect was not seen after the resolution of SIRS. (Shawcross et al., 2004) Activation of astrocytes and microglia through ligation of TLR2 and TLR3 has been shown to result in secretion of IL-6, CXC-10 and IFN-β and differential changes in TLR expression dependent on cell type. (Jack et al., 2005, Lu et al., 2005) Activation of astrocytes and microglia in the central nervous system (CNS) can act as a prelude to CNS inflammation, endothelial activation and margination of activated neutrophils. Release of ROS, NO and defensins (proteases) with subsequent endothelial and cellular damage may contribute to HE (Ramaiah and Jaeschke, 2007, González-Amaro and Sánchez-Madrid, 1999). Researchers have observed that levels of the cytokines IL-6 and IL-18 were found to be significantly higher in patients with minimal-HE. (Montoliu et al., 2009)

Whilst direct evidence for this neutrophil recruitment to the cerebral endothelium is currently lacking, a neutrophil predominant infiltrate is seen in septic encephalopathy, which has been shown to correlate with TNFα levels. (Alexander et al., 2008)

3.7 Innate immune dysfunction in chronic liver failure

3.7.1 Opsonisation

Opsonisation function and subsequent receptor-mediated endocytosis is impaired due to low levels of immunoglobulins and serum complement proteins in cirrhosis. (Runyon et al., 1985, Ono et al., 2004) Furthermore, low ascitic complement levels correlate with decreased bactericidal activity and increased risk of SBP (Such et al., 1988, Runyon et al., 1985). Other proteins involved in innate immunity synthesised by the liver include C-reactive protein (CRP), protein-C and mannose binding lectin (MBL). The exact indication of deficiencies in these proteins in acute or chronic liver failure is unclear at present.

3.7.2 Neutrophils in cirrhosis

Neutrophil express a primed phenotype in patients with cirrhosis and AoCLF including 'shedding' of L-selectin (CD62L), upregulation of surface CD11b, CD64 and CD49d (VLA-4 not present on resting neutrophils), and down regulation of CD16. (Fiuza et al., 2002, Rosenbloom et al., 1995, Hayashi et al., 2003) In neutrophils from patients with sepsis this primed phenotype is associated with enhanced expression of the intracellular transcription gene, NF-kB. (Nakamori et al., 2003) A similar phenotype is seen in patients with sepsis without liver disease. (Brown et al., 2006) However, prolonged neutrophil LPS exposure may result in down-regulation of cytokine/chemokine receptors and intracellular signalling pathways promoting receptor–effector pathway uncoupling and endotoxin tolerance. (Parker et al., 2005) Indeed chronic endotoxemia directly induces decreased expression of the TNF receptor by neutrophils in alcohol-related liver disease. (von Baehr et al., 2000)

Abnormalities in neutrophil function in cirrhosis have been observed since the 1970's with documented abnormalities including an increased neutrophil adherence both to nylon fibres and endothelial cells (Altin et al., 1983, Feliu et al., 1977, Fiuza et al., 2002), reduced chemotactic response, decreased aggregation and locomotion (Yousif-Kadaru et al., 1984, Feliu et al., 1977, Rajkovic et al., 1984) and decreased capacity for phagocytic and OB. (Feliu et al., 1977, Fiuza et al., 2002, Rajkovic and Williams, 1986) Neutrophil dysfunction is further exaccerbated by the presence of superimposed inflammation such as in acute alcoholic hepatits and during infection. (Rosenbloom et al., 1995, Mookerjee et al., 2007b) and correlates with advancing liver disease. (Tritto et al., 2011)

Furthermore patients with cirrhosis develop neutropaenia as a consequence of hypersplenism, which is further exacerbated by accelerated apoptosis, shortening the neutrophil life-span (Kusaba et al., 1998) Enhanced neutrophil apoptosis is mediated through the Fas/Fas ligand pathway. (Kusaba et al., 1998, Liles et al., 1996) Alcohol excess can cause direct myelotoxicity further reducing circulating neutrophil numbers (Ballard, 1997) and has been observed to inhibit neutrophil chemotaxis. (Lister et al., 1993)

3.7.3 Monocytes / peripheral blood mononuclear cells (PBMCs)

Neutrophils act in concert with PBMCs in mediating the innate immune response to infection. Abnormal monocyte function is found in cirrhotic patients with spreading across endothelial surfaces, chemotaxis, phagocytosis and microbial-killing mechanisms all being reduced. (Hassner et al., 1979) Monocytes from patients with AoCLF have reduced antigen presentation capabilities due to reduced surface expression of human leukocyte antigen-DR (HLA-DR) with similar findings being seen in sepsis and ALF. (Wasmuth et al., 2005, Antoniades et al., 2006) Furthermore, FcγR expression and function is reduced on mononuclear cells in alcohol-related liver disease inhibiting clearance of opsonised bacteria. (Gomez et al., 1994) The LPS-induced monocyte production of TNFα is higher in Child-Pugh C than in Child-Pugh B cirrhosis, suggesting that the severity of liver disease has an impact on the innate immune response. (Galbois et al., 2009) Hyper-responsiveness of *ex vivo* monocytes has been reported in primary biliary cirrhosis (PBC). (Mao et al., 2005) The absence of induction of the inhibitor IL-1 receptor-associated kinase M in LPS-stimulated cirrhotic monocytes may in part explain TNFα hyperproduction. (Tazi et al., 2006) Furthermore, the normal generation of the anti-inflammatory cytokine IL-10 by monocytes in response to LPS appears to be defective in cirrhosis. (Le Moine et al., 1995)

3.8 Impaired adaptive immunity in cirrhosis

Lymphocyte function is impaired in cirrhosis with defects in both B- and T-cells. B-cell hyper-reactivity, particularly in alcohol-related cirrhosis, results in enhanced production of secretory IgA from unmethylated cytosine-guanine dinucleotide (CpG) activated B-cells, acting through the TLR9 pathway. (Massonnet et al., 2009) There is expansion of activated CD4+ T-cells (Albillos et al., 2004) including Th17 cells which have a pro-inflammatory phenotype. (Pelletier et al., 2010) Enhanced suppressor T-cell function is observed despite unchanged suppressor T-cell numbers in alcohol and viral liver disease. (Alexander et al., 1983)

Recently mitogen activated CD4+ T-cell metabolic reactivity has been reported in hepatitis C cirrhosis and cirrhosis of other causes. (Yee et al., 2013) Abnormalities in natural killer (NK) cell function are seen in alcohol-related liver

disease. (Laso et al., 1997) The involvement of cellular immune responses not only predisposes to infection either directly in a similar fashion to immunoglobulin abnormalities but also generates a chronic immune perturbation of the innate and adaptive immune response resulting in abnormal responses following infection.

3.9 Summary of immune dysfunction in cirrhosis

The immunopathology in cirrhosis encompasses the entire immune system with involvement of humoral, cell-mediated and cellular immune functions. The hierarchical importance of these defects is currently unclear and many patients with cirrhosis continue to survive infection, suggesting that an adequate response can be generated to infection in many cases. Indeed, it is the over-exuberant and often inappropriate pro-inflammatory response to infection that often leads to organ dysfunction with poor outcomes. This is clearly exacerbated if the immune system is encountering difficulty in eradicating the infecting microbe. Neutrophils are cells central to the defence against microbial infection and the documented abnormalities are likely to be prime candidates to explain the apparent paradox of the co-existence of concomitant immunosuppression and immune hyper-responsiveness in liver cirrhosis.

3.10 Defects in the innate immune system in acute liver failure (ALF)

ALF is less studied than cirrhosis but shares many features of both chronic liver disease and severe sepsis including high plasma levels of pro- and anti-inflammatory cytokines and immune paresis. Monocytes demonstrate a state of functional deactivation characterised by a reduced expression of HLA-DR and a degree of endotoxin tolerance with enhanced IL-10 production. (Antoniades et al., 2006) Complement levels are markedly reduced (the liver being the principle site of complement synthesis) resulting in reduced opsonic capacity and decreased chemoattraction. (Wyke et al., 1980) A reduction in the production of ROS and phagocytic capacity of neutrophils from patients with ALF has been reported both *in vivo* and *in vitro*. (Clapperton et al., 1997, Rolando et al., 2000a) Fibronectin, another hepatically-derived opsonin involved in Kupffer cell clearance, is markedly reduced in ALF (Imawari et al., 1985) and indeed Kupffer cell activity is depressed as

demonstrated by the reduced uptake and clearance of indium-labelled micro-aggregated albumin in ALF. (Canalese et al., 1982) Low levels of HDL, which binds and neutralises the bioavailability of LPS and LTA, are low in ALF due to impaired hepatic synthetic function. (Zambon et al., 1995, Etogo-Asse et al., 2012) Low levels of HDL are also associated with a poor outcome in ALF and AoCLF. (Etogo-Asse et al., 2012)

3.11 Mechanisms for neutrophil dysfunction in liver failure

Recent studies in alcohol-related liver disease suggest that defects in neutrophil function are affected through serum factors such as endotoxin levels. (Mookerjee et al., 2007b) Subsequent *ex vivo* removal of LPS by passing cirrhotic serum through a polymixin B column decreased the spontaneous OB (SOB) and improved phagocytic function of healthy neutrophils. Shawcross et al. propose an alternative mechanism with hyperammonemia and hyponatraemia functioning synergistically to cause neutrophil swelling, akin to cerebral astrocytes, and impaired phagocytosis. (Shawcross et al., 2008b)

Levels of tuftsin, a neutrophil modulator of phagocytosis, are reduced in patients with cirrhosis, with an associated increased incidence of bacterial infection. (Trevisani et al., 2002) Neutrophils from patients with cirrhosis also display a markedly reduced activity of phospholipase C (PLC). (Laffi et al., 1993, Garfia et al., 2004) PLC is stimulated following ligation of G-protein coupled receptor activation (e.g. via chemokine/fMLP receptors) on the neutrophil cell membrane. PLC and phosphinositide3-kinase hydrolyse and phosphorylate phosphatidylinositol 4,5-bisphosphate, generating inositol 1,3,4-triphosphate (IP3) and DAG. An important down-stream response of DAG in neutrophils is activation of PKC, which phosphorylates $p47^{phox}$, leading to activation of NADPH oxidase, thus explaining the reduced production of superoxide following neutrophil stimulation observed by some groups. (Garfia et al., 2004)

Neutrophils undergo down-regulation and apoptosis following signals from LPS-activated regulatory T-cells mediated through TLR activation (Lewkowicz et al., 2006) implying a role for the adaptive immune system in conditions of chronic endotoxemia in limiting overzealous neutrophil activation by decreasing neutrophil

survival. Over-activation of these cells in liver cirrhosis may contribute to the immune paresis observed and merits further investigation.

3.11.1 Ammonia and p38-MAPK pathways in the pathogenesis of neutrophil dysfunction

Serum ammonia levels are raised in ALF and liver cirrhosis due to a reduced urea synthesis capacity and porto-caval shunting. It has recently been shown by Shawcross et al. that ammonia at concentrations commonly seen in acute and chronic liver failure cause neutrophil swelling resulting in a reduced phagocytic capacity and high SOB (Shawcross et al., 2008b). These results were replicated in patients with cirrhosis with induced hyperammonaemia suggesting a direct toxic effect of ammonia on neutrophils, an effect exacerbated by the presence of hyponatraemia. The p38-MAPK intracellular signalling pathway appears to be important in mediating this ammonia-induced dysfunction (Figure 7). Phosphorylation of p38-MAPK, a key osmoregulator and regulator of inflammatory gene expression, including TNFα, IL-1 and IL-12 can lead to activation of neutrophil apoptotic pathways. (Zu et al., 1998, vom Dahl et al., 2001) The susceptibility of neutrophils to ammonia is used to the advantage of some microorganisms and has been shown to be a virulence factor in *helicobacter pylori* infection and human periodontal infection. (Mayo et al., 1997, Niederman et al., 1990)

Ammonia has also been shown to depress neutrophil chemotaxis by decreasing the affinity of chemokine receptors for fMLP, a key bacterial chemotaxin and activator of neutrophils. (Coppi and Niederman, 1989) Additionally, it has been recognised for many years that ammonia can impair neutrophil energy metabolism, which is reliant on the conversion of glutamine to glutamate. The phosphate-dependent glutaminase enzyme that catalyses this reaction is inhibited by low levels of ammonia. (Sbarra and Karnovsky, 1959) Taken together these findings suggest a critical role for ammonia in the impairment of neutrophils in liver failure, which leads to an increased propensity to infection and the subsequent development of HE and MODS.

3.11.2 Role of CARS in neutrophil dysfunction in liver failure

CARS is the counter-regulatory homeostatic mechanism which accompanies the SIRS response and results in increased circulating levels of anti-inflammatory cytokines and mediators aimed at preventing overwhelming inflammation and ultimately resolving infection. It is defined by persistently elevated circulating levels of IL-4, IL-10 and TGF-β, and by impairment in cellular immune function. This is demonstrable in the phenotypic and functional changes that occur in monocytes, resulting in their deactivation, an event central to the development and evolution of CARS. Here a decrease in pro-inflammatory cytokine secretion is accompanied by loss of antigen presenting capability, while production of IL-10 is increased. The point of transition from a homeostatic response to pathological state of CARS characterised by excessive immunosuppression and increased predisposition to infection, has not been clearly defined.

IL-10 release can be favoured by the action of catecholamines that are known to contribute to the altered responsiveness of circulating leukocytes. In a murine model of haemorrhagic shock β-adrenoceptor blockade allowed for a partial restoration of responsiveness of blood leukocytes to LPS in terms of TNF production.(Asehnoune et al., 2006)

3.12 Neutrophils, SIRS, sepsis and organ-failure

SIRS is the systemic consequence of an exaggerated inflammatory response distant to the infectious or inflammatory insult. Patients with liver failure frequently display a SIRS phenotype. SIRS can progress through a series of clearly defined syndromes to septic shock with subsequent progression to MODS and death. SIRS is characterised by the generation of both pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) and anti-inflammatory cytokines (IL-10 and TGFβ); an excess of either component of the response is associated with organ failure and mortality.

Innate immune dysfunction present in liver failure, particularly in the presence of pre-primed immune cells, leads to an exaggerated ‘cytokine storm’ when PAMPs such as LPS and LTA are encountered. This is elegantly demonstrated by the re-challenging of PBMCs and neutrophils from patients with cirrhosis, with LPS, generating high levels of pro-inflammatory cytokines particularly TNFα and IL-6. (Byl et al., 1993,

Wang et al., 2000) Overwhelming activation of primed circulating neutrophils in sepsis can lead to ALI, AKI and septic encephalopathy. (Brown et al., 2006, Awad et al., 2009) Systemic activation of neutrophils is seen to extend neutrophil survival and impedes margination across the vasculature. (Brown et al., 2006) Endothelial damage in organs distant to the initial insult lead to neutrophil recruitment, (Nuytinck et al., 1988) with subsequent generation and release of ROS, and granule contents (e.g. the serine proteases elastase and MMP) at these sites. (Smith, 1994) In addition NO generation from tissue macrophage and PBMCs additionally contributes to the oxidative stress and vasodilatation of sepsis particularly in the splanchnic bed in cirrhosis. Indeed, NO metabolite concentrations correlate with LPS levels in cirrhosis. (Guarner et al., 1993, Lee et al., 2010)

This process of neutrophil over-activation in response to infection can develop rapidly and if left unchecked, can rapidly lead to endothelial dysfunction, hypotension and MODS. Interestingly, this situation can be exacerbated acutely following antibiotic therapy by increasing LPS levels and pro-inflammatory cytokine release. (Prins et al., 1995)

As discuss above the development of deteriorating hepatic function and extra-hepatic organ-failure in patients with cirrhosis as a consequence of an acute event such as SBP and variceal bleeding is termed AoCLF. (Sen et al., 2002) Clinically up to a third of patients who are admitted due to acute deterioration in cirrhosis develop extra-hepatic organ failure with an associated in-hospital mortality between 53-59%. (Jalan et al., 2012b, Shawcross et al., 2012) There is some debate as to whether AoCLF represents a distinct syndrome or just the natural consequence of infection in cirrhosis. One of the important features of the concept of AoCLF is the potential for hepatic re-compensation and reversal of organ failure following successful management of the precipitating event, and it is this feature that sets it apart from the gradual decline observed in end-stage liver disease.

SIRS and liver failure are co-dependent phenomena with the severity of the underlying liver disease determining the outcome from SIRS. The presence of SIRS can precipitate variceal bleeding, HE and be detrimental to survival

3.13 Hepatic recruitment of neutrophils in acute liver injury

The liver is highly metabolically active and is dependent on a ready supply of oxygen delivery through the blood. It is also the first port of contact for ingested toxin and through the cytochrome P450 system can generate toxic adducts from drugs which can directly lead to hepatotoxicity (e.g. acetaminophen) or secondary immune-mediated toxicity for example drug-induced liver injury (DILI). In chronic liver failure, neutrophils not only contribute to the increased propensity to infection seen but have been directly implicated in the liver injury observed particularly in AAH.

Neutrophils can be rapidly recruited to the liver following systemic or local exposure to cytokines such as TNFα, IL-1β, IL-6, chemokines and ROS from Kupffer cells. (Ramaiah and Jaeschke, 2007) These pro-inflammatory mediators prime and activate neutrophils leading to recruitment in the hepatic sinusoids and post-sinusoidal venules. Upon receiving appropriate secondary chemotactic signals neutrophils migrate into the hepatic parenchyma, facilitated through β_2-integrin mediated adhesion along chemokine gradients, particularly CXC-chemokines such as IL-8, macrophage-inflammatory protein-2 (MIP-2), KC, CINC-1 and osteopontin produced from biliary epithelial cells. (Koh et al., 2007) However, neutrophil recruitment to the liver sinusoid does not require β_2-integrin adhesion molecules. Neutrophil accumulation within the sinusoid or post-capillary venule does not typically cause liver injury and the liver is generally immunotolerant. (Chosay et al., 1997) Hepatic parenchymal apoptosis and necrosis can develop directly or indirectly, through the generation of chemokines or through direct cell-cell contact through sieve plates triggering neutrophilic extravasation. (Ramaiah and Jaeschke, 2007) Complement activation, the generation of ROS and lipid peroxidation products from necrotic cells can enhance neutrophil recruitment. (Jaeschke, 2000) Once recruited neutrophils generate further chemokines such as LT-B4 and IL-8. (Jaeschke, 2003) For neutrophils to cause cytotoxicity they need to receive further chemokine signals and indeed, CXC chemokine release does not always lead to neutrophil extravasation and there are requirements for localisation, gradient, timing during the inflammatory response and the balance compared to other chemoattractants. (Dorman et al., 2005) Neutrophils respond to apoptotic and necrotic cells, and apoptotic signals can trigger neutrophilic infiltration; blocking apoptosis by pan-caspase inhibitors prevented neutrophil extravasation into the parenchyma. (Jaeschke et al., 1998)

3.13.1 Neutrophil mediated parenchymal liver damage

Once neutrophils have infiltrated the liver parenchyma they directly bind to ICAM-1 on hepatocytes via β_2-integrins; subsequently they can degranulate releasing potent serine proteases and ROS such as H_2O_2 and hypochlorous acid. (Jaeschke, 2006) ROS can diffuse into hepatocytes leading to increased intracellular oxidant stress and mitochondrial dysfunction. Depleted levels of reduced glutathione within the cells due to the concomitant ingestion of alcohol or other drugs can aggravate hepatic injury. (Jaeschke et al., 1999) Oxidants appear to be the dominant mediators of damage due to the importance of glutathione and that inhibition of NADPH oxidase protects against neutrophil mediated cytotoxicity. (Gujral et al., 2004, Ho et al., 1996) Neutrophil-derived proteases are involved in transmigration and cell-toxicity but can also promote the inflammatory response by the processing of pro-inflammatory cytokines, chemokines and growth factors.

Neutrophils often exacerbate the liver damage caused by toxins causing injury in the liver but especially in prolonged neutrophilic hepatitis such as AAH but in acetaminophen-induced liver injury this is less prominent and neutrophils may be passive and limit extension of the area of injury. Factors such as calpains and deoxyribonuclease (DNase)-1 are subsequently released from necrotic cells can promote ongoing tissue injury. (Limaye et al., 2003, Napirei et al., 2006) Necrotic cells release HMGB-1, which further promotes neutrophilic hepatitis and tissue damage through activation of TLR4 on macrophages and cytokine production. (Scaffidi et al., 2002) This phenomenon has been documented in ischaemia-reperfusion, endotoxemia, and AAH and there is some evidence for it in acetaminophen-induced liver toxicity. (Ramaiah and Jaeschke, 2007)

IL-17 and IL-23 are critical mediators of neutrophil recruitment and migration through the induction of granulopoiesis and production of G-CSF, IL-6, TNFα and neutrophil chemokines. (Ley et al., 2006, Stark et al., 2005) IL-17 levels are increased in alcohol-related liver disease with increased production from PBMCs, with their CD4+ T-cells displaying an IL-17 phenotype. IL-17 secreting cells including neutrophils and T-cells contribute to liver inflammatory infiltrates as well as foci of alcoholic hepatitis. (Lemmers et al., 2009) Enhanced IL-17 production is likely to contribute to neutrophil recruitment and liver inflammation. Furthermore, abnormal T-cell function contributes to the hepatic inflammation with Lin et al. recently showing that Th1 predominant alcohol

dehydrogenase-specific T-cell responses correlate with disease severity in alcohol-related cirrhosis, especially in active alcohol drinkers. (Lin et al., 2013)

Neutrophil dysfunction with high SOB and reduced phagocytic capacity has been reported in patients with alcohol-related cirrhosis and AAH. (Mookerjee et al., 2007b) Alcoholic hepatitis is defined histologically by an intense neutrophilic infiltrate within the liver and these cells are purported to play a key role in the associated hepatocellular damage. (Bautista, 2002) Both of these conditions are associated with a high incidence of bacterial infection, with sepsis and endotoxemia.

Acute overdose with acetaminophen is the leading cause of hyper-acute liver failure in the UK and USA. (Bernal et al., 2013, Lee, 2004) Acetaminophen causes liver injury in overdose following the generation of the reactive metabolite N-acetyl-para-benzoquinone imine (NAPQI) through the cytochrome2E1, an alternative detoxification route to the usual conjugation and biliary excretion that metabolises up to 90% of the drug. NAPQI is further detoxified by glutathione (GSH). (Nelson and Bruschi, 2003) Accumulation of NAPQI leads to reactions with a sulfhydryl group, leading to acetaminophen protein adducts. Acetaminophen protein adduct accumulation, particularly in mitochondria, leads to mitochondrial oxidative stress and cell necrosis. (Jollow et al., 1973)

Acetaminophen-induced liver damage leads to an intense neutrophil hepatic parenchymal infiltrate. However, the precise role of these neutrophils appears to be in a supportive role to mop up damaged cells and cell debris. (Ramaiah and Jaeschke, 2007) Neutrophil recruitment mirrors the development of liver injury but inhibition of neutrophils through adhesion molecules or inhibitors of NADPH oxidase does not protect against hepatotoxicity. (Lawson et al., 2000) Some investigators suggest that neutrophils may exacerbate the liver injury in some conditions. The role of systemic neutrophil activation in the accompanying MODS typically seen in acetaminophen-induced liver failure has been poorly studied.

3.14 Neutrophils as therapeutic targets in liver failure

Neutrophils present attractive targets for modulatory therapies in liver failure both to prevent initial infections but to also ameliorate the pro-inflammatory response that leads to organ failure. The recruitment of activated neutrophils during an inappropriately vigorous response to an inflammatory stimulus into the liver and other extra-hepatic

organ circulations such as the brain and kidney, can lead to the clinical syndromes of AAH, HE and HRS. However, a paradox exists in developing pharmacotherapeutic agents that enhance the antibacterial properties of neutrophils as these can further exacerbate the exaggerated pro-inflammatory response and *vice versa*. Potential strategies include reducing bacterial translocation from the gut, reducing neutrophil priming and activation (e.g. cytokines and ammonia), inhibition of neutrophil adhesion to activated endothelium in susceptible organs, enhancing neutrophil phagocytosis and burst, and promoting neutrophil apoptosis. The ultimate modulatory therapies may prove to be extra-corporeal liver support devices, plasmapheresis and LT.

3.14.1 Therapies affecting bacterial translocation

Modulation of the intestinal microbiota has been the mainstay of treatment for HE for many years but recently more novel agents have become available to reduce bacterial translocation of LPS and other bacterial activators of TLRs in addition to ammonia, which is principally generated from colonic bacteria. (Mencin et al., 2009) Many of these drugs have been used as therapies in cirrhosis with little understanding of the mechanism of action. Laxatives, such as the non-absorbable disaccharide lactulose and lactilol have been used as first-line agents in the treatment of HE for many years. (Als-Nielsen et al., 2004) Lactulose is thought to act by decreasing colonic pH, potentially reducing bacterial translocation and absorption of ammonia as well as preventing bacterial overgrowth of the small bowel. Reduced bacterial endotoxemia and decreased neutrophil priming and activation may well have direct effects on p38-MAPK phosphorylation in addition to ammonia reduction. Effects of non-absorbable disaccharides are modest and indeed strong supportive randomised-control trial (RCT) data is lacking. (Als-Nielsen et al., 2004)

Superior agents at reducing bacterial translocation are non-absorbable antibiotics. Selective gut decontamination has previously been used as a strategy for the treatment of HE using agents such as neomycin, colistin sulphate and amphotericin. These have been largely abandoned due to risks of developing of resistant bacteria and due to unpleasant toxicities such as ototoxicity and nephrotoxicity. This strategy is also employed for the secondary prophylaxis against infection following an episode of SBP or in recurrent HE. Antibiotic prophylaxis with non-absorbable antibiotics such as norfloxacin, co-trimoxazole and more recently rifaximin-α has been shown to reduce bacterial

translocation and the incidence of SBP. (Gines et al., 1990, Singh et al., 1995, Hanouneh et al., 2012, Kalambokis et al., 2012) Concern has however been raised regarding the use of fluoroquinolones as this may lead to the development of resistant organisms and *Clostridium Difficile* infection. Rifaximin-α has been found to be particularly effective at reducing episodes of HE and acute hospital admissions with a low risk of bacterial resistance. (Bass et al., 2010) Interestingly norfloxacin has been shown to abrogate neutrophil superoxide generation and neutrophil apoptosis 24-hours following a single dose. (Zapater et al., 2009) In this study serum and ascitic levels of pro-inflammatory cytokines and NO were seen to reduce with the lowest levels correlating with peak serum norfloxacin levels. Trimethoprim-sulphamethoxazole did not modulate cytokine production suggesting a norfloxacin specific modulation of neutrophil function and inflammatory cytokine production possibly achieved through selective gut-decontamination. (Zapater et al., 2009) Furthermore selective gut-contamination, achieved through pre-treatment with the non-absorbable antibiotic norfloxacin, prevents AKI in a rodent model of cirrhosis following an LPS challenge (inflammatory insult) and is associated with increased renal expression of TLR4. (Shah et al., 2012)

Probiotics have been shown to reduce bacterial translocation and are associated with a range of improvements in liver function, prevention of infection and prevention of HE. (Liu et al., 2004a) Stadlbauer et al. recently showed that probiotics could restore neutrophil phagocytic capacity in alcohol-related cirrhosis. (Stadlbauer et al., 2008) They postulated that the improvements in neutrophil dysfunction seen may be due to reduced levels of IL-10 and lower TLR4 expression, as levels were significantly elevated in *ex vivo* neutrophils from patients compared to healthy controls prior to probiotic therapy. After probiotic therapy reduced expression of these molecules was seen to occur. Further trials have not seen a benefit in these agents in reducing complications of cirrhosis such as infection and HE. (Holte et al., 2012, McGee et al., 2011, Shukla et al., 2011) The combination of these agents with antibiotics is therefore now being explored.

3.14.2 Reduced neutrophil adhesion

A novel way of reducing neutrophil adhesion has been shown to have promise in a model of ALI where a canine hookworm derived beta-2 integrin inhibitor was transfected using liposomal transfer into a murine model of ALI. This resulted in reduced neutrophil

transmigration into the alveolar spaces and prevented lung vascular injury. (Zhou et al., 1998)

3.14.3 Reduced neutrophil priming

Therapies that target the underlying pro-inflammatory cytokine milieu and reduce neutrophil activation indirectly currently offer more prospects at present. On the simplest level, the infusion of albumin in patients with cirrhosis has been shown to reduce the incidence of HRS in SBP and improve survival. (Sort et al., 1999) This appears to be above and beyond its role as a plasma expander and a comparison with an alternative colloid, Dextran-70 has demonstrated reduced levels of plasma renin and improved endothelial dysfunction in the albumin treated group compared to the colloid. (Planas et al., 1990) Albumin possesses an exposed thiol group that act as an antioxidant allowing it to scavenge transition metal ions and free radicals. The use of albumin in SBP in combination with antibiotics significantly reduces levels of TNFα, IL-6 and NO in ascites and serum. (Chen et al., 2009)

Interestingly, extracorporeal liver support systems and albumin dialysis have been shown to enhance cytokine clearance (Di Campli et al., 2005, Stadlbauer et al., 2006) but do not appear to alter serum levels possibly due to a high rate of production. As yet albumin-dialysis has not been shown to confer a survival benefit. Haemofiltration can achieve a 22% reduction in median arterial ammonia concentrations over 24-hours and normalise hyponatraemia, both of which potentially modulate neutrophil function but its effects in this setting has yet to be determined. (Slack et al., 2014)

Plasmapheresis is the removal, treatment and return of plasma from the circulating blood. The procedure is used to treat a number of inflammatory conditions such as Guillain-Barre syndrome and haemolytic uraemic syndrome where circulating factors are present. The process has the potential to remove a significant quantity of cytokines, PAMPs and DAMPs, which may have beneficial immunomodulatory effects. Studies have shown however, that circulating concentration of cytokines seem to be unaltered probably due to the high levels of on-going production. (Nakae et al., 2002) However, high volume plasmapheresis has been shown to alleviate severe HE in patients with ALF with improvement in systemic haemodynamics. (Larsen et al., 1996) Plasmapheresis is currently being evaluated in acetaminophen-induced ALF where LT is not an option.

Ongoing attempts at removal of PAMPs have shown potential benefit in sepsis with successful reduction in the levels of endotoxin and IL-6 using an apheresis system based on DEAE-cellulose. (Bengsch et al., 2005) An albumin replacement system with a novel endotoxin ligation (ARSeNEL) component has been developed and is undergoing trials by the liver failure group at UCL. Improved survival, reduced endotoxin levels and improved intracranial pressure index has been observed in early studies and a clinical trial is being planned. (Ryan et al., 2013)

Leukopheresis involves extracorporeal removal of leukocytes from the blood either through centrifugation or by passage of blood through an adsorptive system. In each system, venous blood is removed in a continuous flow, anti-coagulated, processed to deplete the leukocytes and returned to the circulation. These remove a mixture of cells but mainly granulocytes and lymphocytes. These systems have been approved for use in inflammatory bowel disease following a RCT and a number of case series. (Tominaga et al., 2013, Hanai et al., 2003) Intriguing supportive evidence that down-regulation of primed neutrophils may be a therapeutic goal is suggested by studies showing leucodepletion improves respiratory and renal dysfunction in sepsis. (Treacher et al., 2001) This may be of particular benefit as it targets the dysfunctional circulating neutrophils which are implicated in organ dysfunction while leaving neutrophils recruited to sites of inflammation unaffected. This has shown success in inflammatory bowel disease where activated neutrophils play a key role in tissue damage but its use in liver disease has only been reported in a case of refractory AAH. (Ota et al., 2009)

Excitement surrounds the prospect of small molecules that modulate TLR4 signalling which can potentially downregulate neutrophil activation and other cellular immune responses. (Mullarkey et al., 2003) Early data indicate that they can reduce LPS-stimulated cytokine release in healthy volunteers and are awaiting phase III clinical trial results. Neutrophil TLR9 expression in ALF and cirrhosis serves as a useful biomarker that may differentiate patients that develop high grade HE. High baseline neutrophil TLR9 expression is associated with a pro-inflammatory milieu and may explain the propensity to develop infection and hasten the development of HE. (Manakkat Vijay et al., 2012) Therefore TLR9 antagonists may be of therapeutic value in restoring bacterial defence mechanisms.

3.14.4 Immunosuppressive drugs

Neutrophils can be suppressed by corticosteroids and these drugs perturb multiple steps in the immune activation of all inflammatory cells including neutrophils, with reduced antigen presentation, cytokine production and lymphocyte proliferation. Peripheral neutrophil counts increase in response to corticosteroid administration due to reduced margination of cells and increased export of neutrophils from the BM. Neutrophil adhesion is reduced which reduces recruitment into sites of inflammation. Corticosteroids block a key step in the production of prostaglandins and leukotrienes, through inhibition of phospholipase-A2; these are important neutrophil chemotactic molecules. IL-1 production is also inhibited. More long-term effects of corticosteroids are seen though the decreased action of T-cells and reduced clonal proliferation of T-cells through the inhibition of IL-2 (T-cell growth factor) production. Circulating neutrophils showed increased spontaneous burst and decreased L-selectin expression in this cohort. Upon stimulation, neutrophils produced large quantities of IL-8 and TNFα. Plasma levels of pro-inflammatory cytokines are high in AAH but IL-10 levels are barely detectable in comparison to patients with alcohol-related cirrhosis. (Taieb et al., 2000) Treatment with corticosteroids normalised cytokine profiles and neutrophil function.

The use of immunosuppressant drugs particularly in the setting of AAH has been a rather vexed question for many years. AAH occurs in the setting of chronic liver disease with up to 80% of patients showing cirrhosis on subsequent liver biopsy. Alcoholic hepatitis is an intense neutrophilic hepatitis that is manifested by liver inflammation, hepatocyte injury and fibrosis and can lead to a SIRS and decompensation of the chronic liver disease; in the most severe form this can be associated with a high mortality. (Forrest et al., 2005) Patients with mild disease are seen to spontaneously recover whilst the use of potent immunosuppressive drugs is a high-risk gambit in the cohort of patients with severe disease who are at high risk of overwhelming sepsis. As yet corticosteroids have not been shown to demonstrate a clear benefit in AAH, possibly due to heterogeneity in the patient selection. (Mathurin et al., 2011) Stratification according to disease severity [Maddrey's modified discriminant function >32 (Maddrey et al., 1978)] can result in improved 30-day mortality especially in those with HE but is still associated with 40% mortality. (Phillips et al., 2006) The recent large multi-centre STOPAH trial confirmed a reduced 28-day mortality but this did not reach significance at 90-days and 1-year. (Thursz et al., 2015)

Pentoxifylline is a methylated xanthine derivative and is a competitive non-selective phosphodiesterase inhibitor. Pentoxifylline increases intracellular cyclic AMP, activating PKA resulting in the inhibition of TNF and leukotriene production. This can reduce inflammation and activation of neutrophils and monocytes. In placebo-controlled trials of pentoxifylline in AAH, the mortality rate decreased in the active therapy arm (26% versus 46%). This was not due to improved liver function but the reduced incidence of renal dysfunction in those treated with the drug. (Akriviadis et al., 2000) Modulation of neutrophil function would be a potential mechanism through which this effect may be mediated either through direct suppression of neutrophil function or secondary to reduced TNFα levels; a key pro-inflammatory cytokine in alcoholic hepatitis, although the precise mechanism of action is unclear. However, the STOPAH trial has recently cast doubt on the clinical effectiveness of pentoxifylline showing that survival in groups treated with 28-days of monotherapy was unchanged compared to placebo groups and no additional benefit was seen when combined with prednisolone. (Thursz et al., 2015)

TNFα is a key mediator in AAH and directly targeting this cytokine with inhibitory monoclonal antibodies such as the anti-TNF monoclonal antibody infliximab has been considered an attractive strategy. A pilot study of 20 patients with biopsy-proven alcoholic hepatitis who were treated with corticosteroid and either infliximab or placebo was published in 2002. (Spahr et al., 2002) Infliximab therapy resulted in improved discriminant function and IL-8 levels at 28-days. Infliximab has also been shown to reduce peripheral neutrophil counts in AAH. (Tilg et al., 2003) However, a subsequent RCT comparing corticosteroids to infliximab was stopped early due to an unacceptable high mortality from sepsis in the infliximab arm. (Naveau et al., 2004) Entanercept the soluble TNF-receptor fusion protein does not appear to have a clinical effect in AAH. (Boetticher et al., 2008) The effects of potent immunosuppressant drugs such as steroids and anti-TNF antibodies demonstrate the difficulties with suppressing neutrophil function. This appears to be beneficial in some (particularly when receiving more moderate doses) but others develop overwhelming infection as these agents exacerbate the immune dysfunction already present.

3.14.5 Neutrophil apoptosis

Targeted therapies have produced mixed results in sepsis such as G-CSF (Nelson et al., 1998, Root et al., 2003) and antagonism of pro-inflammatory cytokines (Netea et al.,

2003) have not shown benefit unless neutrophil patients have low percentages of immature cells and low concentrations of G-CSF. (Ishikawa et al., 2000) G-CSF has been shown to improve neutrophil phagocytic capacity in patients with ALF. (Rolando et al., 2000b) However, G-CSF therapy has been shown to benefit patients with AoCLF who were treated with 12 doses of G-CSF over a month and showed improvement in liver failure scores with reduced septic episodes and an outcome at 2-months associated with increased circulating numbers of neutrophils and CD34+ cells (bone-marrow derived stem cell). (Garg et al., 2012) The authors' postulate that G-CSF may improve survival in AoCLF through neutrophil activation and improved hepatocyte regeneration, however, neutrophil phenotype or functional analysis was not performed which would have provided additional insight into the findings.

3.14.6 Novel therapies targeting specific neutrophil functions

Hypothermia is a therapeutic measure used in ALF to prevent cerebral oedema. Intraoperative hypothermia has been shown to reduce the production of ROS and phagocytosis by neutrophils *ex vivo* during conditions where the core temperature was reduced to 33-37,° (Wenisch et al., 1996) suggesting that stabilisation of neutrophil burst may be a desired therapeutic goal. It displays many beneficial effects on brain water and intracranial hypertension relating to decreased brain ammonia, cerebral blood flow, mediators of inflammation and oxidative stress. (Jalan et al., 1999, Larsen et al., 1996) There has however, been some concern that hypothermia may be detrimental to immune function impairing defence mechanisms against microbial challenge. (Stravitz and Larsen, 2009) Hypothermia has been shown to decrease neutrophil functions such as oxidative killing and phagocytosis *in vivo and in vitro.* (Wenisch et al., 1996, Akriotis and Biggar, 1985) However, D Shawcross has shown that moderate hypothermia (33 ºC) prevented the ammonia-induced activation of resting neutrophil oxidative burst *in vitro*, without impairing the ability of the neutrophils to phagocytose opsonised *E. coli* or undergo stimulated oxidative burst and thus kill bacteria. (Shawcross et al. unpublished data)

Modulation of intracellular second messenger systems such p38-MAPK may be considered to be a therapeutic target of the future as selective modulation may allow fine control over neutrophil functions in different circumstances. Shawcross et al. demonstrated that ammonia-induced neutrophil swelling could be abrogated to some

extent by treating with p38-MAPK agonist isoproterenol. (Shawcross et al., 2008b) This not only prevented neutrophil swelling but also normalised phagocytosis. However, selective p38-MAPK antagonists such as SB203580 inhibit the ammonia-induced increase in spontaneous oxidative burst. As our understanding advances, other second messenger targets may be explored such as modulators of ERK, JNK and PI3K. Furthermore, other osmoregulators such as the aquaporin molecules, may provide alternative therapeutic targets. (Hao et al., 2012)

Chapter 4 - Aims and Objectives of the study

This thesis sets out to investigate the neutrophil characteristics present in acute and chronic liver failure and the putative role played by neutrophils in organ dysfunction in this condition. I wish to challenge the traditional view that liver failure is a condition associated with an "immuneparesis", instead suggesting that neutrophils actively undergo defective dysregulation of their anti-microbial effector functions, thus leading to an increased propensity to infection and playing a critical role in the development of organ failure, especially in the presence of complicating microbial infection. It is only by understanding the mechanisms that underlie the altered behaviour of neutrophils in this condition that new, effective and targeted therapies can be developed to reduce the unacceptably high mortality from sepsis associated with liver failure.

4.1 Hypothesis.

This study aimed to test the following hypotheses:

1. Neutrophil swelling, impaired phagocytosis and spontaneous oxidative burst are present in patients with liver failure and contribute to increased susceptibility to infection.
2. Arterial ammonia contributes to neutrophil dysfunction and swelling, mediated through changes in the p38-MAPK pathway [see Figure 4-1].
3. Abrogation of the neutrophil dysfunction seen in liver failure can be achieved by pre-incubation of *ex vivo* neutrophils with specific modulators of p38-MAPK.

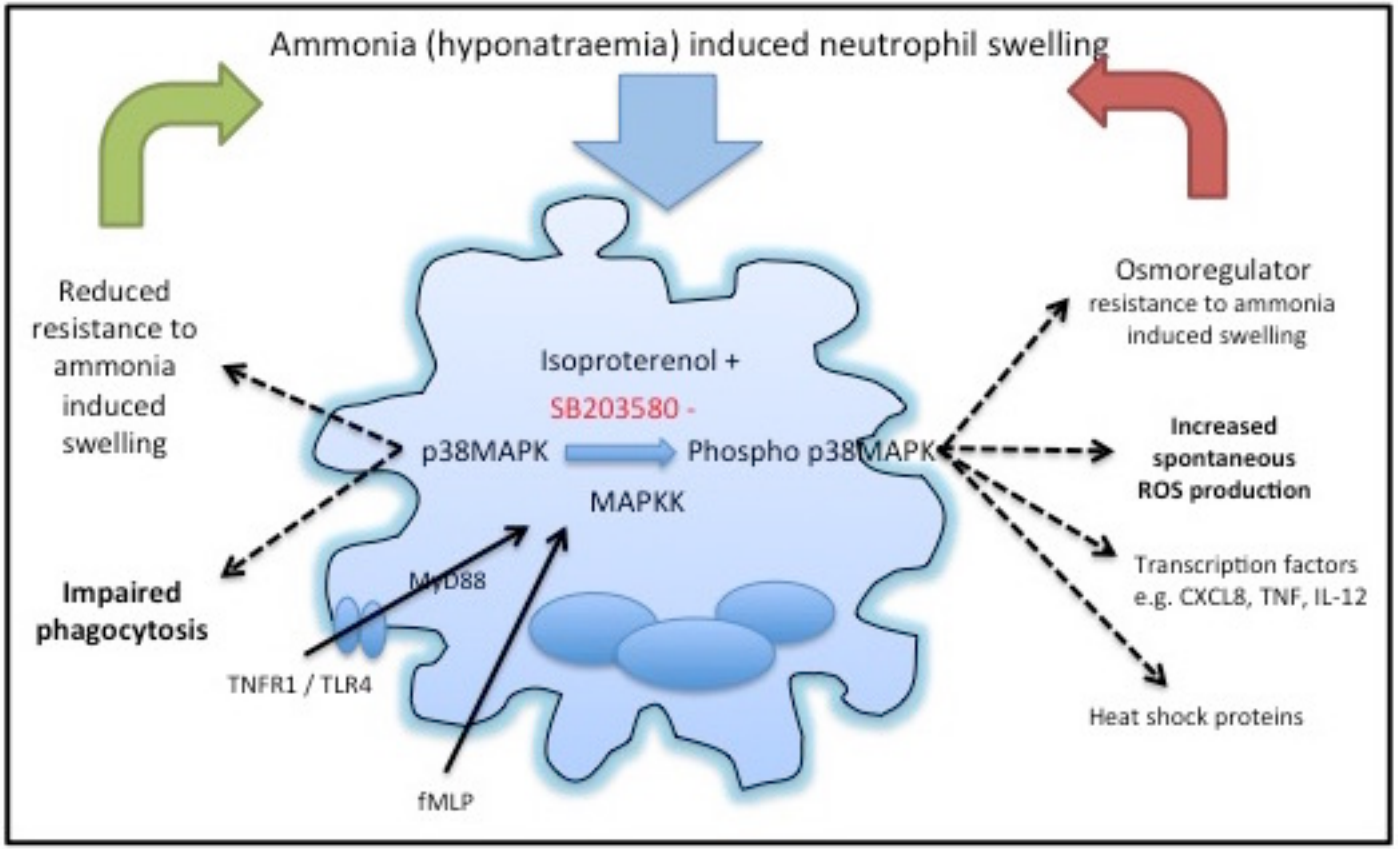


FIGURE 8-1 HYPOTHESIS MODEL - AMMONIA INDUCED NEUTROPHIL SWELLING AND COMPENSATORY ACTIVATION OF P38-MAPK SYSTEM.

p38-MAPK becomes activated following phosphorylation by MAP kinase kinase. SB203580 is a specific inhibitor of p38-MAPK activation and the β-adrenergic agonist isoproterenol increased phosphorylated p38-MAPK through activation of cAMP-dependent protein kinase but not cJNK. (Moule and Denton, 1998)

4.2 Studies to be performed and their objectives.

4.2.1 Study to characterise neutrophil morphology and function in acute and chronic liver failure

Neutrophil morphology (cell volume and transmission electron microscopy), chemotaxis, phagocytic function, spontaneous and *E.coli* stimulated oxidative burst (SOB and ESOB) were compared between patients and healthy controls in order to characterise the neutrophil defects in acute and chronic liver disease. Comparison of neutrophil function and morphology between patients with liver failure and patients with severe sepsis requiring ICU admission, determined if the neutrophil phenotypes are comparable. It is important to determine if neutrophil activation is occurring in a similar fashion to that observed in sepsis, possibly due to endotoxemia, or if unique or more profound changes occur in the context of liver failure, which may suggest alternative pathways of neutrophil priming and activation.

Comparison with measures of disease severity, inflammation, arterial ammonia, and/or hyponatraemia allowed determination of the factors that correlate with neutrophil function confirming the findings of previous studies. Secondly, associations with the severity of liver disease/injury were investigated. In addition, associations with other plasma-derived factors such as pro- and anti-inflammatory cytokines, immunoglobulins, complement and lipids, all of which may be potentially immunomodulatory, were sought in order to aid in the understanding of the implications of these phenomena.

The role of liver disease aetiology (e.g. alcohol-related liver disease and chronic viral hepatitis) and the presence of neutrophil dysfunction were investigated as previous studies have shown increased neutrophil dysfunction especially in patients with alcoholic hepatitis. The association of neutrophil dysfunction with significant patient outcomes such as infection, variceal bleeding, HE and death were investigated to determine if neutrophil dysfunction could act as a predictor of such events.

In ALF, correlation between neutrophil phenotype/function and change over time was determined and compared with severity of liver injury and measures of associated organ failure to determine if neutrophil dysfunction predicts poor outcome that might help refine selection for LT.

4.2.2 Study to determine the contribution of neutrophil swelling and dysfunction to the increased susceptibility to infection seen in patients with liver failure

Short-term and long-term follow-up of these cohorts documenting clinical end-points such as incidence of inflammation, infection, organ dysfunction and survival were performed to answer this question. My hypothesis was that patients with more severe neutrophil dysfunction develop more infections (e.g. SBP) and complications of their liver disease including variceal bleeding, HE and AKI. In addition I wanted to determine if neutrophil dysfunction is an independent risk factor for death over and above severity of liver failure. This may identify patients at high risk in whom antibiotic prophylaxis could be considered

4.2.3 Study to determine the change in neutrophil morphology and function during the development of HE or AoCLF in patients with liver failure

Patients admitted with HE or who developed HE during their in-patient stay had blood sampling performed during the acute episode of HE and (where possible) on convalescence to determine if there were any differences, as both ammonia and inflammation have been shown to play a role in the development of this complication. A second sub-group of patients who had developed complications of their cirrhosis leading to MODS requiring ICU support (AoCLF) were also characterised. In this population I wanted to know the effect of infection and an increasing inflammatory milieu on neutrophil phenotype/function and outcome.

4.2.4 Study to determine how ammonia-reducing therapies such as haemofiltration and liver transplantation impact on neutrophil function?

To document the effects of *in-vivo* ammonia lowering by haemofiltration and LT on neutrophil swelling and function, samples were obtained before and 48 hours after the initiation of continuous veno-venous haemofiltration (CVVH) or post-LT. As ammonia or hyponatraemia-induced neutrophil swelling is believed to be an important mediator of neutrophil dysfunction I hypothesised that normalisation of these factors by such therapies would abrogate neutrophil dysfunction.

4.2.5 Study to determine whether arterial ammonia concentration contributes to neutrophil swelling and dysfunction through changes in the p38-MAPK pathway

Quantification of phosphorylated and total p38-MAPK levels were measured by Cytokine Bead Array (CBA) assay (Becton Dickinson, UK) and subsequently determined by Fluorescence activated cell sorting (FACS) using ACAP array software (Becton Dickinson, UK). The hypothesis that ammonia impairs neutrophil function by inducing cell-swelling resulting in elevated levels of phosphorylated p38-MAPK (the activated form) leading to activation of homeostatic mechanisms to correct cell swelling and induction of transcription of inflammatory genes within neutrophils was tested by correlating blood ammonia concentration with neutrophil swelling and function.

4.2.6 Study to investigate the effect of modulators of the p38-MAPK pathway on neutrophil function

In order to investigate the effect of modulators of p38-MAPK on neutrophil function *ex-vivo*, analysis was performed on isolated neutrophils at baseline, following ammonia exposure and following incubation with 1μM isoproterenol a p38-MAPK agonist or 10μM 4-(4-Flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole [SB203580] a p38-MAPK antagonist.

Chapter 5 - Materials and Methods

5.1 Study design

A cross-sectional case-control cohort study was devised. Patients with ALF and cirrhosis were prospectively studied. Neutrophil morphology, chemotaxis, phenotype, phagocytosis, SOB and ESOB were determined and compared to healthy and septic disease controls. Patients were recruited between October 2008 and August 2010. Patients with ALF, AoCLF and severe sepsis were recruited from the ICU. Patients with liver cirrhosis were either recruited following admission for LT assessment or following attendance at the hepatology outpatient clinic.

5.2 Patient cohorts - inclusion and exclusion criteria.

Table 5-1 Study groups to be enrolled and sampling protocol.

Study Group	**Aetiology / precipitating factor**	**Number of patients to be recruited**	**Samples to be taken**
Healthy controls	Non-smoker and no alcohol / exercise within 24 hours	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml)
Septic disease controls	Severe sepsis	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml); repeat day 3 and on recovery
Chronic liver disease	Child's A cirrhosis	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml)
Chronic liver disease	Child's B/C cirrhosis	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml) repeat post-LT
HE (grade I-II)	No active sepsis	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml) day1; repeat on recovery from HE (grade 0)
AoCLF	Sepsis / variceal bleed / HE grade (III-IV)	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml) day 1; repeated on day 3; post LT or one recovery
Acute / Subacute liver failure	Paracetamol and Non-paracetamol	24	3 x Li^+ heparin (9 ml) & 1 x EDTA (2 ml) day 1; repeated on day 3; post LT or on recovery
Total		**168**	

5.2.1 Control cohorts

Healthy age- and sex-matched healthy volunteers with no history of liver disease were recruited to the healthy control group. Their alcohol intake was <20 g/day and they had not drunk alcohol or exercised excessively in the 24-hours prior to blood being drawn.

Patients with severe sepsis, as defined by the presence of a SIRS response [temperature>38°C or <36°C; heart rate >90 beats per minute; tachypnoea > 20 breaths per minute or $PaCO_2$<4.3 kPa; white cell count >12 X 10^9/L or <4 X 10^9/L; or the presence of >10% immature neutrophils], (Bone et al., 1992) with radiological or laboratory evidence of infection and end-organ failure were recruited from the ICU to the sepsis disease control group. Blood sampling was performed within 48-hours of admission and on subsequent days, with a convalescent sample prior to discharge [Table 2-1].

5.2.2 Acute Liver Failure Cohorts

ALF is a rare but frequently catastrophic consequence of severe liver injury. Trey and Davidson originally defined the entity in 1970 as the onset of hepatocellular dysfunction in the absence of pre-existing liver disease characterised by coagulopathy and encephalopathy within 8 weeks of the hepatic insult, lasting no more than 26 weeks duration. (Trey and Davidson, 1970) In addition patients were further sub-classified depending on O'Grady's classification (O'Grady et al., 1993) which uses time from appearance of jaundice to the development to HE;

(i) Hyperacute liver failure being defined as a jaundice to encephalopathy time ≤ 7 days which is associated with severe coagulopathy and intracranial hypertension. It is generally associated with a good survival rate without emergency LT.

(ii) Acute liver failure defined as jaundice to encephalopathy time of 8-28 days and is typified by presentation with acute hepatitis B infection. Patients have moderate degrees of coagulopathy, jaundice and intracranial hypertension with generally less favourable outcomes without LT.

(iii) Subacute liver failure has a more indolent course with jaundice to encephalopathy time of 4-12 weeks being commonplace, with severity of jaundice outweighing the coagulopathy. Intracranial hypertension is less common complicating only

approximately 9% of cases, as compared to 20% in acute and hyperacute liver failure. (Bernal et al., 2007) For the purposes of this study ALF was considered as the development of encephalopathy within 28 days of the onset of jaundice and subacute liver failure (SALF) being defined as the development of encephalopathy 4 – 12 weeks from the onset of jaundice (O'Grady et al., 1989). Exclusion criteria were age <18 or >80, evidence of bacterial, fungal or viral sepsis on clinical examination and/or cultures within 48 hours of sampling, malignancy, pregnancy and any coexisting history of immunodeficiency including human immunodeficiency virus (HIV) infection and glycogen storage disease.

Baseline sampling was performed within 48 hours of admission to the ICU and on subsequent days until spontaneous recovery or LT. In those who underwent LT further sampling was performed 72 hours post-LT. Subjects were followed up for 90-days.

5.2.3 Cirrhosis Cohorts

All patients had a diagnosis of cirrhosis made on biochemical, radiological and/or histological findings. Exclusion criteria were age <18, evidence of bacterial, fungal or viral sepsis on clinical examination and/or cultures within 48 hours of recruitment (apart from the AoCLF study group), malignancy, pregnancy and any coexisting history of immunodeficiency including HIV and glycogen storage disease. Patients were recruited to the following groups: Mild to moderate stable cirrhosis (Child-Pugh A and B) (Pugh et al., 1973); Advanced stable cirrhosis (Child-Pugh C); Hepatic Encephalopathy (West-Haven grade I-II managed in a hospital ward environment) (Harold and Milton, 1979); AoCLF, defined as the development of deteriorating hepatic function and extra-hepatic organ failure in patients with cirrhosis as a consequence of an acute event such as SBP and variceal bleeding. (Sen et al., 2002, Moreau et al., 2013)

Sampling was performed at baseline, following the development of HE or sepsis with matched convalescent samples, and following LT. Subjects were followed up for 12 months or until LT [Table 2-1].

5.3 Sample size and power calculation

Based on power calculations using previous *in-vitro* and *ex-vivo* data from pilot data (Figure 5-1 and 5-2), a 20% reduction in neutrophil function from 90% to 70% was anticipated. Comparing a proportion of 0.9 to 0.7 using the binomial proportion comparison and assuming a two-sided test, alpha 0.5 and power of 80%, gives a sample size of 24 required in each group.

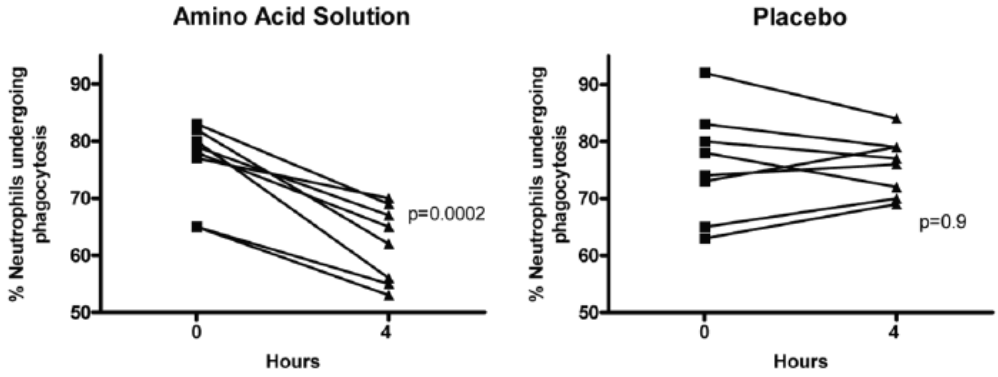


Fig. 1. A graphical representation to illustrate the reduced phagocytosis at 4 hours in each of the eight patients administered the amino acid solution compared to the eight given the placebo solution. A paired *t* test showed this to be significant at $P = 0.0002$.

FIGURE 5-1 REDUCTION IN NEUTROPHIL PHAGOCYTOSIS 4 HOURS POST INGESTION OF AMINO ACID SOLUTION OR PLACEBO IN PATIENTS WITH CIRRHOSIS

(Shawcross et al., 2008b)

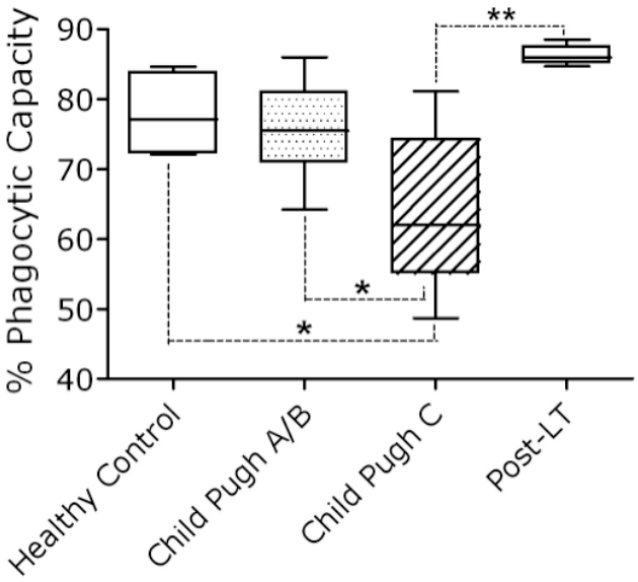


FIGURE 5-2 GRAPH SHOWING PERCENTAGE UPTAKE OF OPSONISED *E COLI* BY NEUTROPHILS IN HEALTHY CONTROLS AND PATIENTS WITH LIVER CIRRHOSIS PRE AND POST-LIVER TRANSPLANTATION

(Taylor et al., 2009)

5.4 Consent and data collection

The study was performed in accordance with the declaration of Helsinki and ethical permission was granted from the North East London Research Ethics committee (Ref No 08/H0702/52). Following the obtaining of fully informed consent/assent, clinical, biochemical and physiological data were collected. Data included tobacco and alcohol use, arterial ammonia (μmol/L), serum sodium levels (mmol/L), differential leucocyte count ($x10^9$), complement and lipoprotein levels. The Glasgow Coma Score (GCS) [Table 5-2] (Teasdale and Jennett, 1974), grade of HE according to the West-Haven criteria [Table 5-3] (Harold and Milton, 1979), and admission SIRS score were also calculated. [Table 5-4] (Bone et al., 1992) A number of organ failure scores were also quantified including the CPS [Table 5-5], (Pugh et al., 1973) model of end-stage liver disease score (MELD) (Kamath et al., 2001), United Kingdom End-Stage Liver Disease score (UKELD) (Barber et al., 2011), Sequential Organ Failure Assessment (SOFA) Score [Table 5-6] (Vincent et al., 1996) and the Acute Physiology and Chronic Health Evaluation (APACHE) II score. (Knaus et al., 1985) Length of ICU stay, survival and

number of days requiring vasopressors (terlipressin was not handled as a vasopressor in this study), ventilation or renal replacement therapy were also analysed.

Details of potentially immunomodulator therapies such as N-acetyl cysteine, corticosteroids, antibiotics and Pentoxifylline therapy, haemofiltration and plasmapharesis were recorded. The occurrence of infection in the 4 weeks prior to sampling, and in the 6 weeks following sampling, were recorded along with relevant patient outcomes including infections (e.g. SBP, variceal bleeding, HE) and survival (6-months for CLD and 90-days for ALF)

Table 5-2 Glasgow Coma Scale (GCS)

	1	2	3	4	5	6
Eyes	Does not open eyes	Opens eyes to painful stimuli	Opens eyes to voice	Opens eyes spontaneously	n/a	n/a
Verbal	No sounds	Incomprehensible sounds	Inappropriate words	Confused	Orientated	n/a
Motor	No movements	Extensor posturing to painful stimuli	Flexor posturing to painful stimuli	Withdraws to painful stimuli	Localises painful stimuli	Obeys commands

Table 5-3 West Haven Criteria for Semi-quantitative grading of Mental Status

Grade 1	Trivial lack of awareness Euphoria or anxiety Shortened attention span Impaired performance of addition or subtraction. Asterixis can be detected
Grade 2	Lethargy or apathy Minimal disorientation for time or place Subtle personality change Inappropriate behaviour. Obvious Asterixis
Grade 3	Somnolence to semi-stupor, but responsive to verbal stimuli Confusion Gross disorientation
Grade 4	Coma (unresponsive to verbal or noxious stimuli)

Table 5-4 Systemic inflammatory response syndrome (SIRS) score

Systemic inflammatory response syndrome (SIRS) at least 2 of the criteria listed below must be present to diagnose.	
Temperature:	>38 ^{o}C or <36 ^{o}C
Heart rate	>90 beats per minute;
Respiratory rate	>20 breaths per minute or $PaCO_2$ <4.3 kPa [self-ventilating]
White blood cell count (WBC)	>12 x 10^9/L or <4 x 10^9/L or the presence of >10% immature neutrophils
Sepsis is diagnosed when patients meet SIRS criteria, and has a documented or suspected source of infection	
Severe sepsis – sepsis associated with at least one sign of organ dysfunction, including; hypoperfusion, or hypotension lactic acidosis, oliguria, or alteration in mental state.	
Septic shock – severe sepsis with hypotension unresponsive to adequate fluid resuscitation.	

Table 5-5 Child-Turcotte-Pugh score

Parameter	1 point	2 points	3 points
Total bilirubin μmol/L	<34	34-50	>50
Serum albumin g/L	>35	28-35	<28
INR	<1.7	1.7-2.3	>2.3
Ascites	None	Mild	Moderate - Severe
Hepatic encephalopathy	None	Grade I-II	Grade III-IV

Table 5-6 Sequential Organ Failure Assessment (SOFA) Score

	0	**1**	**2**	**3**	**4**
Respiratory PaO_2/FiO_2	>400	≤400	≤300	≤200	≤100
Coagulation Platelets x 10^3	>150	≤150	≤100	≤50	≤20
Liver Bilirubin μmol/L	<20	<32	<101	<204	≥204 or MARS
Cardiovascular Hypotension	No hypotension	MAP<70	Dop≤ 5 or dobu or PDI	Dop>5, epi≤0.1 or norepi≤0.1	Dop>15, epi>0.1 or norepi >0.1 or CASD
Central nervous system GCS	15	13-14	10-12	6-9 or ICP	<6
Renal Creatinine μmol/L or urine output mls/day	<110	≥110	≥171	≥300 or <500	>400 or <200 or RRT

Abbreviations: MARS-Molecular Adsorbent Recirculating System (liver assist device), MAP-mean arterial pressure, Dop-Dopamine, Dobu-Dobutamine epi-epinepherine, norepi-norepinepharine, CASD-Circulatory assist device, GCS-Glasgow coma scale, ICP-Intracranial pressure, RRT-renal replacement therapy.

5.5 Blood sampling

The schedule for blood sampling is outlined in Table 3. Venous blood was collected aseptically either from a large vein without a tourniquet or from indwelling central venous catheters (in those patients on ICU) into heparinised pyrogen-free tubes from patients/volunteers and was immediately placed on ice on a tilt-table and gently mixed for 10 minutes to pre-cool the neutrophils to 0-4 °C. Subsequently neutrophil function tests were performed within 2 hours of blood being drawn. Plasma was obtained by centrifugation at 4500 rpm for 10 minutes at 4 °C, and stored at -80°C for subsequent cytokine determination.

Arterial blood was sampled aseptically in pre-heparinised syringes either from peripheral radial artery puncture or from indwelling radial or femoral catheters.

5.6 Measurement of Blood Ammonia

Plasma ammonia estimation was performed from freshly drawn arterial blood by determination using bedside testing using the Ammonia test Kit II for the PocketChem BA device (Arkay, Inc, Kyoto, Japan). This measures ammonia in a 20 μL blood sample applied to a reagent strip. The continuous measurement range is 7-286 μmol/L and the normal blood ammonia level for healthy adults for this device is less than 54 μmol/L. The device was maintained in accordance with the manufacturer's specification, with daily internal and monthly external calibration. The device is located within the ICU so all samples were processed in under 5 minutes.

5.7 Ex-vivo studies of neutrophil cell surface expression of CD16 and CD11b

Neutrophil cell surface receptor expression was determined by staining with anti-CD16-Phycoerythrin (PE) IgG_1 κ and anti-CD11b-Allophycocyanin-Cyanin 7 (APC-Cy7) IgG_1 κ (both from BD Biosciences) antibodies. Red cell lysis was achieved by the addition of 1mL of FACS lysis solution (BD Biosciences) to 100 μL whole blood and allowed to stand for 10 minutes at room temperature. Cells were then washed with a further 3 ml of neutrophil wash solution (NWS) and centrifuged (5 mins, 1600 rpm, 4 °C) discarding the

supernatant. Cells were then re-suspended in 100 μL of NWS and then stained with anti-CD16-PE IgG_1 κ or anti-CD11b-APC-Cy7 IgG_1 κ and allowed to conjugate for 1 h in the dark at room temperature. Analysis of the samples was performed on a fluorescence-activated cell sorting Canto II analyser (FACSCantoII, Becton Dickinson, San Jose, CA). Neutrophils were gated on forward scatter (FSC) and side scatter (SSC) characteristics and cells staining positive for CD16-PE and CD11b-APC-Cy7 were determined using flow cytometry (see Method 5.9.5 below for neutrophil gating, acquisition and analysis).

5.8. Assessment of neutrophil cell volume

5.8.1. Assessment of neutrophil cell volume using neutrophil forward scatter as determined by flow-cytometry

Assessment of neutrophil cell volume was performed on neutrophils isolated from heparinised whole blood by density centrifugation using Polymorphoprep™ using endotoxin free equipment in a laminar flow cabinet. Polymorphoprep™ (Axis shield, Norway) is a sterile and ready-made solution used for the isolation of the polymorphonuclear granulocytes from the whole blood. It utilises the principle that the PMN cells are more buoyant in density than the mononuclear cells. It contains Sodium diatrizoate 13.8% (w/v) and Dextran 500: 8.0% (w/v) with Density: 1.113±0.001 g/mL, Osmolality: 445±15 mOsm and Endotoxin: < 1.0 EU/ml (Polymorphprep™).

5.8.2 Method for neutrophil isolation using Polymorphoprep™

1. Heparinised venous blood was collected from healthy controls and patients with liver failure. Tubes were kept on ice and placed on a bench rocker prior to assessment of neutrophil apoptosis and samples were analysed within 30 minutes.
2. Five mLs of polymorphoprep™ were placed in a 15 mL falcon tube. Five mL of blood from the lithium heparin tube were carefully layered on top of the polymorphoprep™ using a pipette. Care was taken for the blood not to mix with the Polymorphoprep™.
3. The tubes were centrifuged at 2800 rpm for 35 minutes at 18 °C without using a brake.

4. Three distinct layers were present after centrifugation; two clear leucocyte bands were seen to form in the falcon tube (Figure 5-3) above a sediment band of erythrocytes. The top leucocyte layer consisted of mononuclear cells with the lower leucocyte layer consisting of PMN cells, including neutrophils. The lower leucocyte layer was harvested using a Pasteur pipette. These PMN were diluted by addition of equal volume of 0.45% saline to restore the osmolality.
5. These cells were washed twice with NWS, in a centrifuge at 2500rpm for 5 minutes. It was re-suspended in 60 µL of neutrophil wash solution
6. Neutrophil volume was determined by FACS Diva software, using a method previously reported for hepatocyte cell volume regulation (Carini et al., 1999). Neutrophil median FSC, as an estimation of neutrophil volume, were expressed as arbitrary units after normalization of 100,000 control neutrophils. The intra-assay coefficient of variance was 4.7% and the inter-assay coefficient of variance was 5.4%.

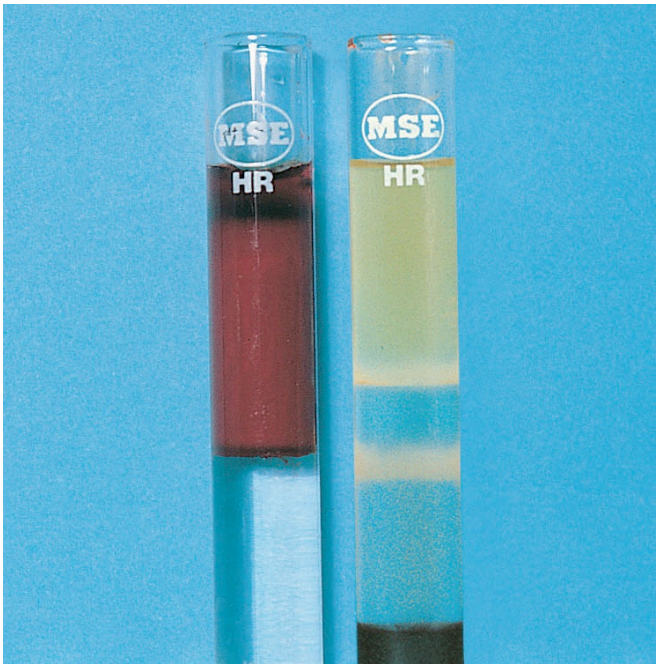


FIGURE 5-3 TUBES SHOWING WHOLE BLOOD LAYERED OVER POLYMORPHOPREP™ BEFORE AND AFTER CENTRIFUGATION AT 2800RPM WITHOUT BREAK.

5.8.3 Neutrophil morphology assessment by transmission electron microscopy

In representative samples from each cohort transmission electron microscopy (TEM) was performed on neutrophils isolated from heparinised whole blood by density centrifugation using Polymorphoprep^{TM} using endotoxin free equipment in a laminar flow cabinet as described above. Neutrophils were subsequently incubated with 20 μL phosphate buffered saline (PBS) or 20 μL of opsonised *E.coli* ($2x10^7$) for 20 minute prior to fixation in glutaraldehyde solution and stored at 4 °C. Subsequently TEM was performed in collaboration with Dr Alice Warley (at the Centre for Ultrastructural Imaging, King's College London) and an independent blinded haematologist evaluated the images, with expertise in morphology (Professor Barbara Bain; St Mary's Hospital) for morphological changes (e.g. phagosome numbers, granule density and evidence of mitochondrial stress). (Robinson et al., 1999) Images were analysed using the Image J software package (imagej.nih.gov/ij/download/)

5.9 Studies to determine neutrophil chemotaxis

Chemotaxis of ex-vivo neutrophils was determined by the Migratest^{TM} (Orpegen Phrama) using the following method:

5.9.1 Leukocyte isolation and assay procedure

1. One ml of heparinised whole blood from either controls or patients was overlayed on top of the neutrophil separation media (12 vials are provide in the kit).
2. The vial was left motionless at room temperature for 40 minutes. At this point the upper of the 3 phases contains the leucocyte rich plasma (LRP). 500 μL of the LRP was aspirated using a pipette, into a 2 μL eppendorf.
3. The fMLP was prepared from stock solution (1:200 dilution) by adding 5 μL to 1mL of incubator buffer
4. The following samples were prepared for each patient or control by pipetting incubator buffer into two wells of a multi-well plate:
    a. Negative control sample - 350 μL Incubator buffer
    b. Positive control - 350 μL of the fMLP working solution

5. Cell culture inserts were placed into both wells of a multi-well plate. 100 μL of LRP were added into each cell culture insert.
6. The multi-well plate is incubated for 30 minutes at 37 °C in a water bath.
7. The cell culture inserts were removed from the multi-well plate. The cell suspensions from both wells of the multi-well plate were added into 2 separate 5 mL Falcon tubes (tubes 1 and 2) and placed on ice. 20 μL of the cells from the cell culture insert of the negative control sample were added to 350 μL of incubator buffer in a third Falcon tube (tube 3).
8. 20 μL of counting reagent was added to each tube, which was vortexed and incubated for 10 minutes in a covered ice bath. Samples were then ready for acquisition.

5.9.2 Flow cytometric acquisition and analysis of neutrophil migrations:

1. Data were acquired after selecting forward scatter area, side scatter area and the Alexafluor 488/FITC channel.
2. A region was set over the leukocytes and counting beads (dot plot diagram of SSC and APC, see Figure 5-4).
3. After activating a gate on region 1, a second region was set around the counting beads (dot plot SSC/FSC, see Figure 5-4); acquisition was then started and ended once exactly 2000 events had been recorded in region 2. This ensured that the amount of granulocytes in the control sample could be compared to the number of granulocytes in the positive control after stimulation with fMLP. A third region around the granulocytes population was set (dot plot diagram SSC/FSC, see Figure 5-4)
4. The following data was recorded from the population in region 3; number of cells (negative control and fMLP stimulated sample) and mean value of the FSC signal (fMLP stimulated sample only)
5. The percentage of activated cells was calculated by the number of cell expressing CD62L (L-selectin). This analysis was performed on a separate histogram (Figure 5-4) generated from region 3 of the fMLP stimulated sample and the CD62L control (Tube 3). On the CD62L control histogram a marker was set for fluorescence so that

less than 15% of cells were activated. This marker was applied to the fMLP-stimulated sample and the percentage of activated cells determined.

6. The inter-assay precision of the assay for 10 samples was 97.0-99.4% activated granulocytes with a mean coefficient of variance 0.2%.
7. The number of activated cells and cell volume was determined by mean forward scatter and compared between the fMLP sample and CD62L-control sample. The proportion of migrating cells was compared between the fMLP and negative control sample (see Table 5-7).

Table 5-7 Flow cytometeric parameters recorded following analysis of the Migratest™

	Tube No. 1	Tube No. 2	Tube No. 3
Parameter 1	Number of cells	Number of cells	-
Parameter 2	-	Mean value of FSC signal	Mean value of FSC signal
Parameter 3	-	Percentage of activated cells	Percentage of activated cells

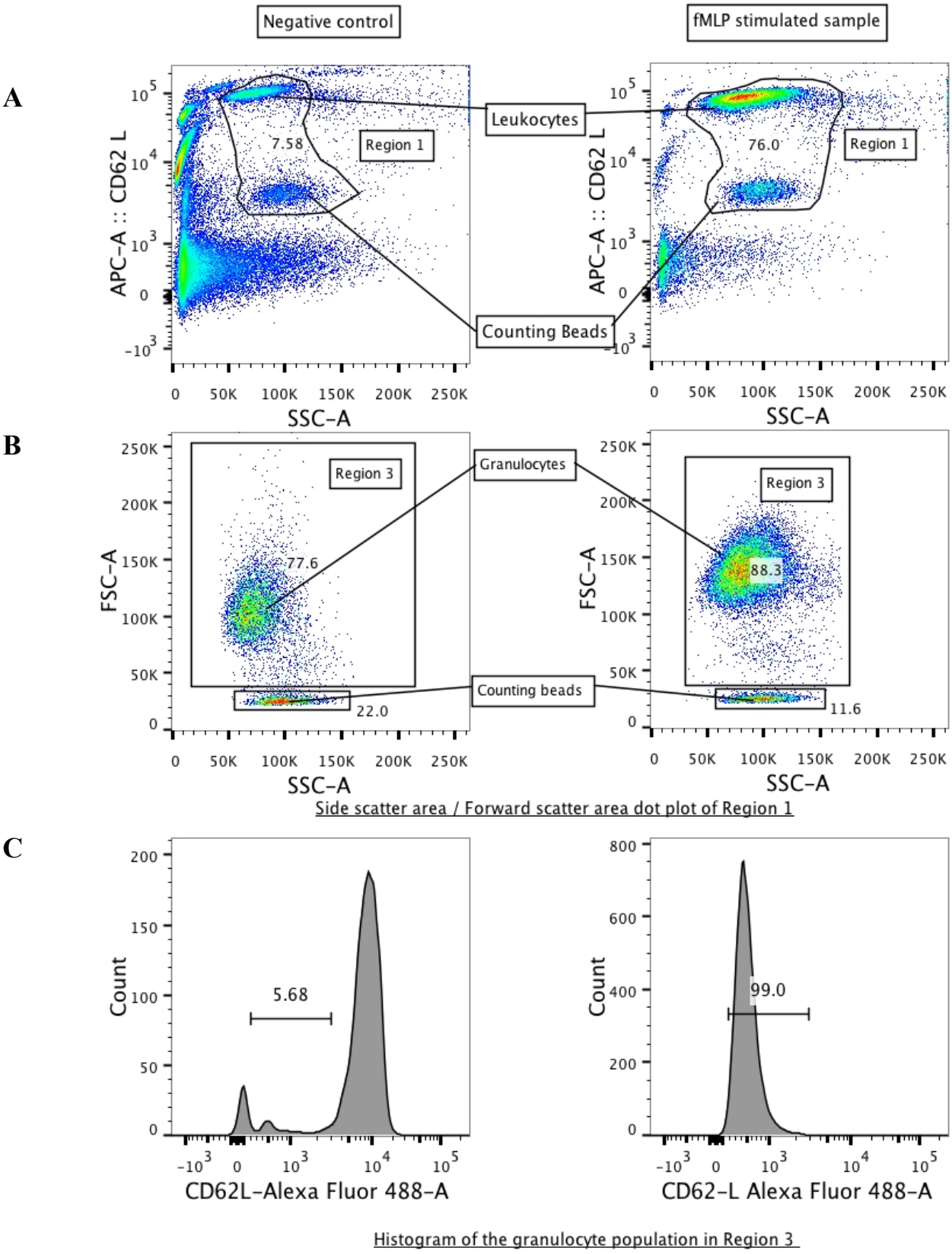


FIGURE 5-4 MIGRATION FLOW PLOTS AND HISTOGRAM ANALYSIS OF CD62L INTENSITY IN A CONTROL AND FMLP ACTIVATED SAMPLE.

5.10 Ex-vivo studies of neutrophil function

All *ex-vivo* studies were performed in pyrogen-free conditions utilising non-pyrogenic 12 x 75 mm falcon tubes. Neutrophil function was examined in neutrophils isolated from whole blood to more closely resemble physiological conditions and to prevent neutrophil activation during separation with all samples being performed in triplicate.

1.22.1 5.10.1 Quantification of neutrophil phagocytosis by flow cytometry using FITC-labelled *Escherichia coli*

Phagocytosis was quantified using the Phagotest (Orpegen Pharma, Heidelberg, Germany). This test allows the quantitative determination of leukocyte phagocytosis in heparinised blood using flow cytometry. The Phagotest uses fluorescein isothiocyanate (FITC)-labelled opsonised *E.coli* bacteria as described previously (Shawcross et al., 2008b). In brief, 100 μL whole blood was mixed with 20 μL of FITC-labelled *E.coli* ($2x10^7$) and incubated in a water bath at 37°C for 20 minutes. Fluorescence of bacteria at the cell surface was quenched using ice-cold trypan blue solution. Samples were washed twice with 3 mL of NWS and centrifuged for 5 minutes at 1500 rpm at 4 °C discarding the supernatant. Red cells were lysed with the addition of 1 mL of FACS lysis solution (BD Biosciences, San Jose, CA) and allowed to stand for 10 minutes at room temperature. Cells were then washed with a further 3 ml of NWS and centrifuged (5 minutes, 1600 rpm, 4°C) discarding the supernatant. To identify neutrophils, cells were stained with anti-CD16-PE IgG_1 κ or anti-CD11b-APC-Cy7 IgG_1 κ and allowed to conjugate for 1 h in the dark at room temperature. Analysis of the samples was performed on a FACSCantoII flow-cytometer (Becton Dickinson, San Jose, CA). Neutrophils were gated on forward and side scatter characteristics and cells staining positive for CD16-PE. Phagocytosis was expressed as the percentage of neutrophils undergoing phagocytosis and the mean of fluorescence intensity (MFI). The inter-assay and intra-assay coefficient of variance for triplicate samples were 1.6% and 10.1%, respectively.

5.10.2 Quantification of neutrophil oxidative burst by flow cytometry using dihydrorhodamine 123

Neutrophil OB was quantified using the PhagoBurst™ test (Orpegen Pharma, Heidelberg, Germany). This test measures the percentage of phagocytic cells that produce ROS with or without *E.coli* as described previously (Shawcross et al., 2008a). In brief, 100 μL of heparinised whole blood was incubated for 20 min with 20 μL of opsonised *E.coli* ($2x10^7$) and either 20 μL of 0.81 μM PMA or 20 μL of 0.5 μM fMLP or 20 μL of NWS, at 37 °C. PMA and fMLP are stimulators of oxidative burst; fMLP through its action on G-protein coupled chemokine receptors and PMA through a direct intracellular effect on Protein kinase C. (Figure 3) PMA is a potent inducer of OB whereas fMLP is a weak stimulator in the absence of prior neutrophil activation. 20 μL of dihydrorhodamine-123 solution was added with a further 20 min incubation at 37 °C. Dihydrorhodamine-123 is oxidised to rhodamine-123 in the presence of reactive oxidant species and gives green fluorescence. Red cells were lysed and neutrophils were washed with NWS prior to analysis. To identify neutrophils, cells were stained with anti-CD16-PE IgG_1 and analysed by FACS. Neutrophils were gated on forward and side-scatter characteristics and, subsequently, the percentage of CD16-positive cells producing ROS was calculated along with the geometric mean fluorescence intensity (GMFI). Samples were analysed in triplicate. The inter-assay coefficient of variance was 4.7 and 2.4 for SOB and ESOB, respectively. The inter-assay coefficient of variance was 5.4% and 4.2% for spontaneous and stimulated OB, respectively.

5.10.3 Method for the preparation of blood samples for the Phagotest/PhagoBurst test

1. The water bath was turned on to 37 °C
2. Blood samples were obtained from the patient – 3 x heparinised samples (9 mL) and 1 x Ethylenediaminetetraacetic acid [EDTA] (24 mL) and placed immediately on ice (approximately 20 mL of either venous or arterial blood). One for phagocytosis and burst tests, one for chemotaxis and one heparinised and EDTA sample for plasma (cytokine analysis by enzyme linked immunosorbent assay [ELISA]).

3. The plasma ammonia was immediately checked using the Ammonia test Kit II for the PocketChem BA device located in liver intensive care unit (see above).
4. Falcon tubes (Becton Dickinson, San Jose, CA) were labelled as per tables 5-8 and 5-9 below:
    i. Phagotest™ - 3 x A per patient and one set of controls tubes per experiment: B, C, D and E (healthy control).
    ii. PhagoBurst™ test -3 x 1 and 3 x 2 per patient and one set of control tubes per experiment B: 5-8 (healthy control).
5. One litre of NWS was prepared in an autoclaved bottle. One vial of supplied powder (Phagotest™ and PhagoBurst™ kits) was dissolved in 1000 mL of double distilled water and refrigerated at 4 °C [NWS is a buffered isotonic salt solution provided with the Phagobust™ and Phagotest™ kits which contains chloracetamide and ethylendiaminetrtraacetic acid sodium salt].
6. One mL of NWS was added to the substrate bottle (PhagoBurst™ test).
7. The opsonised *E.coli* vials (one unlabelled [PhagoBurst] and one FITC labelled [Phagotest]) were removed from the fridge and allowed to equilibrate to room temperature.
8. The fMLP and PMA were diluted to a working concentration by adding 5 μL of the stock solution into an appropriately labelled 1.5 mL eppendorf tube and diluted with 1 mL of NWS, vortexed and kept on ice (made fresh daily).
9. The lysing solution, containing diethylene glycol and formaldehyde, was diluted to a working concentration by adding 5 mL of lysing solution to 45 mL of double distilled water in a 50 mL Falcon tube (BD), and kept at room temperature (can be stored at 4 °C).
10. 100 μL of blood was added to pre-labelled Falcon tubes from step 4.
11. 20 μL of NWS / *E.coli* / PMA / fMLP were added to the relevant tubes see Table 5-7 (Phagotest) and Table 5-8 (PhagoBurst test) and left in the water bath for 20 min.
12. The quenching solution (tryptan blue) was placed on ice (already constituted within Phagotest™ box).
13. 20 μL of substrate were added to the PhagoBurst™ test tubes 1,2,3,4 and 6 (NOT 5 and 7) and placed back in the waterbath for a further 20 min.

14. Meanwhile 100 μL of chilled quenching solution were added to the Phagotest™ tubes A-D (The quenching solution fixes the cells preventing any further uptake of bacteria).
15. The Phagotest™ tubes A-D were washed by adding 2-3 mL of chilled NWS and centrifuged at 1600 rpm for 5 min at 4 °C.
16. The supernatant was aspirated off and step 15 repeated.
17. Tubes 1-7 were then removed from the water bath.
18. For red cell lysis 1 mL of lysing solution was added to all tubes and left for 10 min at room temperature, They were gently mixed by aspirate up and down gently using the pipette.
19. If the samples were incompletely lysed, step 18 was repeated.
20. Two mL of NWS was added to all tubes and centrifuged at 1600 rpm for 5 min at 4 °C before the supernatant was removed.
21. 20 μL of anti-CD16-PE and 5 μL CD11b-APC–Cy7 were added to the appropriate tubes, and left in a darkened cupboard for 20 minutes at room temperature (See Table 5-7 and 5-8).
22. The tubes were then re-washed with NWS and centrifuged at 1600 rpm for 5 min at 4°C.
23. The supernatant was then removed and re-suspend in 500 μL of NWS ready for FACS analysis.
24. **Trypan Blue Exclusion:**

    400 μL of whole blood underwent 2 lysis steps with 1 mL lysing solution as per step 18 above. It was then reconstituted to 400 μL and add 100 μL of trypan blue was added. 10 μL was then placed onto a haemocytometer slide and 4 quadrants were counted and multiply by 4.

Table 5-8 Falcon tube contents for each patient sample in the Phagotest™

Tube number	Heparinised Blood	*E.coli*-FITC	Anti CD16-PE	Anti CD-11b-APC-Cy7
3 x A (patient sample)	100 µL	20 µL	20 µL	5 µL
B (Anti-CD-16 control)	100 µL	NWS 20µL	20 µL	NWS 5µL
C (Anti-CD11b control)	100 µL	NWS 20µL	NWS 20µL	5µL
D (Double negative)	100 µL	NWS 20µL	NWS 20µL	NWS 5µL
E (*E.coli*-FITC control)	100 µL	20 µL	NWS 20µL	NWS 5µL

Table 5-9 Falcon tube contents for each patient sample in the PhagoBurst™ test

Tube Number	Blood	Step 11 addition	Substrate	Anti-CD16-PE	Anti-CD11b APC-Cy7
1-Stimulated burst	100 µL	Opsonised *e.coli* 20 µL	20 µL	20 µL	5 µL
2-Resting burst	100 µL	NWS 20 µL	20 µL	20 µL	5 µL
3-PMA	100 µL	PMA 20 µL	20 µL	20 µL	5 µL
4-FMLP	100 µL	FMLP 20 µL	20 µL	20 µL	5 µL
5-Anti-CD16 control	100 µL	NWS 20 µL	NWS 20 µL	20 µL	NWS 5 µL
6-Anti-CD11b-control	100 µL	NWS 20 µL	NWS 20 µL	NWS 20 µL	5 µL
7-Rhodamine control	100 µL	NWS 20 µL	20 µL	NWS 20 µL	NWS 5 µL
8-Double negative	100 µL	NWS 20 µL	NWS 20 µL	NWS 20 µL	NWS 5 µL

5.10.4 Method for flow-cytometer set-up on the FacsCanto II (BD, San Jose, CA):

1. The cytometer was turned on and the doors opened.
2. The fluidics cart was checked
    a. Waste (empty)
    b. Shutdown fluid (adequate)
    c. Cleaning fluid
    d. Sheath fluid
    e. Bubble catcher was checked and bled especially if a component on the cart had been changed.
3. The computer was turned on and logged on to FACS DIVA software. Under the cytometer menu the "fluidics start-up' was selected and the doors closed.
4. Under the cytometer menu select "cleaning modes" and "de-gas flow-cell" were selected (the flow-cell was checked visually for the presence of bubbles).
5. The cytometer set-up and tracking (CST) performance check was ran:
    a. If running the instrument for the first time a CST baseline check was performed – Three drops of CST beads were placed in 500 μL FACS flow.
    b. Otherwise a daily performance check was run with one drop of CST beads in 350 μL FACS flow (ensuring the CST beads had been vortexed for 5 seconds).
6. The CST outputs were checked. The PE channel Coefficient of variance (CV) gives a good idea of optical performance with a good and robust percentage CV being between 0.1-0.2 with an excellent machine reaching 0.3. However the exact value is not so important but rather the change over time. The optical background (scattered laser light by the sheath fluid) was checked and if significantly increased from the previous reading this suggested that the flow cell was dirty which was managed by a long clean. See Table 5-10 for examples of typical photomultiplier tubes (PMT) voltages on an optimised machine.
7. The CST programmed was exited and DIVA software re-entered.
8. Use CST settings was selected.
9. "New experiment" was then selected from browser.
10. "Tube 1" was then highlighted.
11. The cytometer window gate extension under "Parameters" was then checked [ideally 7 μs].

12. The fluorochromes involved in the experiment were then selected.
13. The fully stained sample (not beads) was then checked and voltage adjustments for FSC and SSC were made by creating a plot of FSC versus SSC. The FSC was usually likely to need to be adjusted as CST was based upon 2 μM and 3 μM beads. Most cell are bigger therefore the FSC was decreased (ballpark average 300 V for cells which are 10-15 μM).
14. The acquisition dashboard was then entered and "Acquire data" (not save data) selected.
15. Thresholds were adjusted to exclude sub-cellular debris (approximately > 20,000, can be done by eye) and following adjustment "acquire" was clicked again to check the plot was not cutting into the sample.
16. Once adjustments to FSC and thresholds had been made they were saved as "application settings" which were automatically updated by CST.
17. The threshold changes were then accepted.
18. For a repeat experiment following a new start-up the relevant application settings were selected to apply.

Table 5-10 Example PMT voltages showing typical values on an optimised FacsCanto II flow-cytometer

Laser		**PMT voltage**	**Bright Bead % Robust CV**
Blue	FSC	542	1.32
Blue	SSC	368	2.84
Blue	E	432	1.75
Blue	D	477	1.41
Blue	B	513	3.08
Blue	A	691	6.15
Red	C	498	2.55
Red	A	584	3.51

Abbreviations; CV: coefficient of variance

5.10.5 Compensation on a FacsCanto II (BD, San Jose, CA) flow-cytometer:

1. Compensation controls were prepared as either cells or capture beads singly labelled with each detection antibody (i.e. PE, FITC and APC-Cy7) [Note separate compensation was performed for Phagotest™ and PhagoBurst™ as rhodamine is not antibody conjugated and so will need to compensate with cells].
2. **For compensation with cells** label the following 5 Falcon tubes were labelled:
   I. Unlabelled lysed blood.
   II. PE labelled lysed blood.
   III. FITC labelled lysed blood or unlabelled *E.coli* incubated stimulated burst sample for rhodamine [tube 7] (emission spectra for FITC and rhodamine overlap and are detected on the same PMT).
   IV. APC-Cy7 labelled lysed blood.
   V. Lysed whole blood labelled with all 3 fluorochromes.
3. 100 μL of control sample blood was added into each tube and 1 mL FACS lysis solution was added and aspirated up and down gently using a pipette. This was left for 10 min. It was then washed twice with NWS and centrifuged at 1600 rpm for 5 min at 4 °C. After each cycle the supernatant was aspirated off and the cells re-suspended in 500 μL NWS.
4. **For Compensation with beads**. The beads were vortexed for 5 s and one drop of beads dispensed with a working concentration of antibody:
   I. 1 x Falcon tube containing blank BD compensation beads.
   II. 1 x Falcon tube containing BD compensation beads and capture beads labelled with antibody (incubated for 20 min at room temp in the dark to fix). Separate samples were needed for each antibody.
   III. Falcon tube containing lysed whole blood labelled with all 3 fluorochromes, were prepared as above in step 3.
5. Under the "experiment" menu "create compensation controls" was selected – a tube list and worksheet was then generated – If using beads for negative (unstained controls) the FSC was changed. The unstained control sample was then selected and acquired.
6. A region was then moved over the bead population and "apply to all" was selected adjusting for cells as required.

7. Once adjustments had been made the data was recorded (default acquisition set-up).
8. The tubes are then acquired sequentially as requested (note include unlabelled compensation beads were included in all samples, for the burst experiments and compensation was set up with cells for PE/rhodamine.
9. Manual adjustments to compensation when using beads was avoided.
10. Once compensation/controls had been acquired the compensation matrix was generated by selecting "calculate compensation controls".
11. The "apply only" option was then selected and a fully stained sample was rechecked (adjusted for FSC if compensation was performed with beads).
12. A return to the global worksheet was then performed and the first tube selected keeping compensation values. The samples were then acquired and the experiment template exported.

5.10.6 Sample gating, acquisition and analysis on the FacsCanto II (BD, San Jose, CA) flow cytometer.

1. Once the FacsCanto II flow cytometer is set-up, CST and compensation was complete a "new experiment" was selected. The previously saved experiment template should be selected and "Create experiment" was chosen.
2. The number of tubes and the fluorochrome present in each tube were then selected.
3. Any fluorochromes not used were deleted from the list and each fluorochrome was labelled with the appropriate antibody.
4. On a global worksheet, a plot: FSC-H/SSC-H dot plot with a linear scale was created and a sample labelled with PE and FITC, such as sample A, was acquired. The threshold was usually set to 20,000 to exclude subcellular debris and the granulocyte population was gated as shown in Figure 5.4 below.
5. 10000 total events were recorded.
6. A population hierarchy was produce by selecting G1 to create a second dot-plot CD16PE/FITC with a logarithmic scale. Using the unstained cells, single labelled CD16-PE and single labelled FITC (beads)/Rhodamine (cells) and control samples second dot plot was gated into quadrants as shown in Figure 5-5 below. Gating for Phagoburst™ and Phagotest™ experiments was performed separately.

Once the gates were set the stats view was edited to display results for each quadrant.

7. Once all the samples had been recorded for both the Phagotest™ and PhagoBurst™ experiments, the same gates were applied across each experiment for consistency.
8. The upper right quadrant shows neutrophils that have not undergone phagocytosis and the upper left shows neutrophils that have undergone phagocytosis of the opsonised *E.coli*. If there are a large proportion of cells in the left and right lower (LL and RL quadrant) this may be due to poor lysis of the sample, incorrect gating or neutrophil apoptosis. Samples with a large numbers in LL and LR quadrants should be examined carefully for quality and results compared to the other triplicate samples and other samples acquired at the same time. Gating may need adjustments, but if significantly different then the sample should be discarded and results not used for further analysis.
9. The average of the triplicate samples should be used for the final analysis (example flow plots of phagocytosis, SOB and ESOB obtained from a healthy control are shown in Figures 5-6, 5-7 and 5-8 respectively whilst corresponding flow plots obtained from a patient with alcohol-related cirrhosis and superimposed alcoholic hepatitis are shown in Figures 5-9, 5-10 and 5-11 respectively)

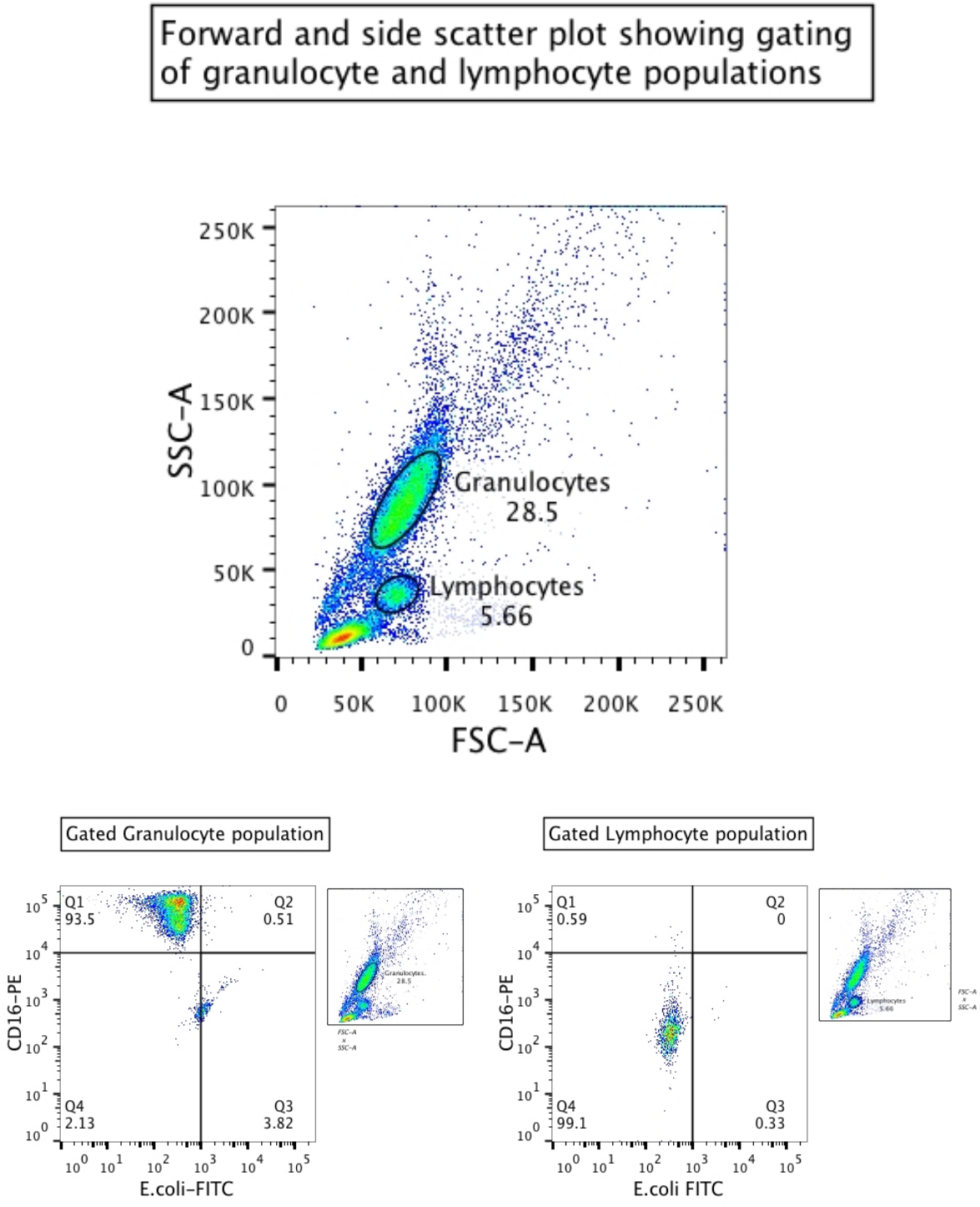


FIGURE 5-5 GATED GRANULOCYTE AND LYMPHOCYTE POPULATION ON A FLOW PLOT OF FORWARD- AND SIDE-SCATTER CHARACTERISTING. PLOTS OF CD-16-PHYCOERYTHERIN AND E.COLI-FITC SHOWING WITHOUT PRE-INCUBATION WITH OPSONISED E.COLI FITC SHOWING GRANULOCYTE AND LYMPHOCYTE POPULATIONS AND GATING STRATEGY SHOWING HIGHER NEUTROPHIL BINDING OF CD16.

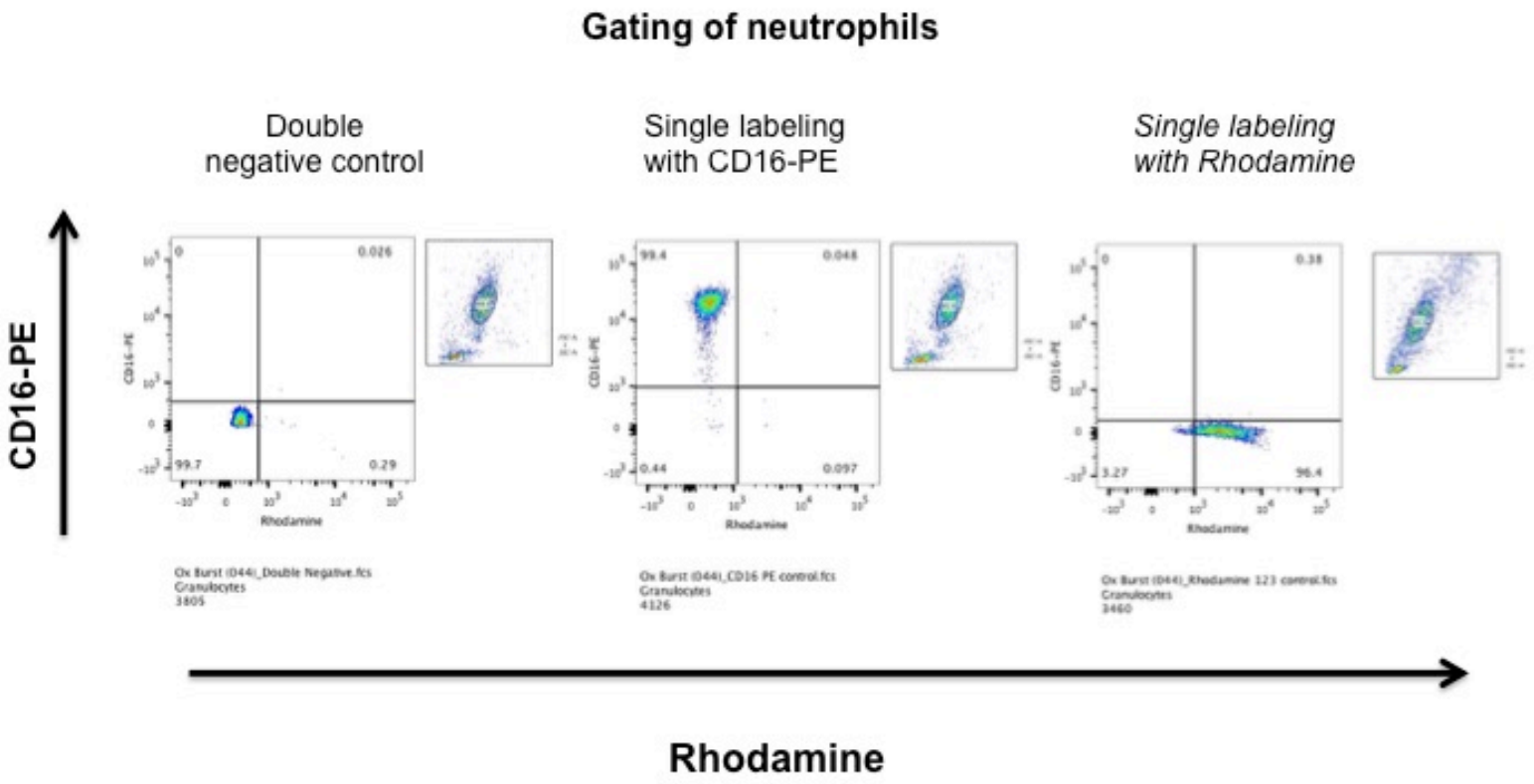


FIGURE 5-6 GATING STRATEGY FOR PHAGOBURST EXPERIMENT USING UNSTAINED AND SINGLE-LABELLED SAMPLES OF CD16-PHYCOERYTHERIN (CD16-PE) AND RHODAMINE.

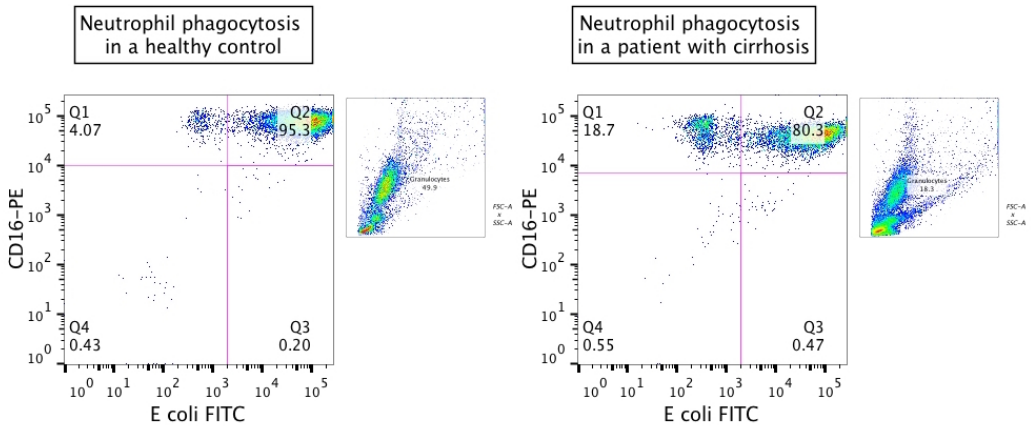


FIGURE 5-7 FLOW CYTOMETRY PLOTS OF A GATED GRANULOCYTE POPULATION WITH ANCESTRY, SHOWING NEUTROPHIL PHAGOCYTOSIS AS DETERMINED BY UPTAKE OF OPSONISED FITC-LABELLED *E.COLI* SHOWING IN A HEALTHY CONTROL AND A PATIENT WITH ALCOHOLIC HEPATITIS (AH) SHOWING.

Q1-non-phagocytosing neutrophils and Q2-Phagocytosisng neutrophils. .

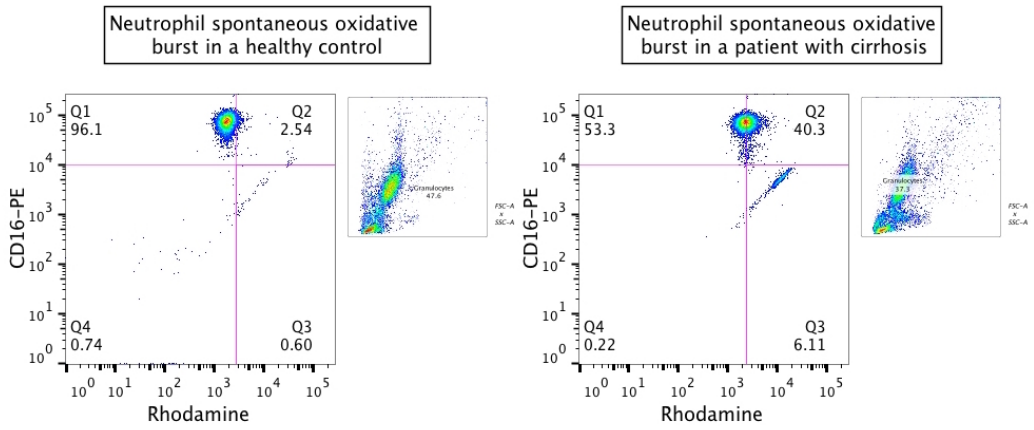


FIGURE 5-8 NEUTROPHIL BURST IN NON-*E.COLI* EXPOSED CELLS WITH OXIDATION DETECTED BY THE INTENSITY OF RHODAMINE-123 FLUORESCENCE DETECTED ON THE FITC CHANNEL IN A HEALTHY CONTROL AND PATIENT WITH ACUTE ALCOHOLIC HEPATITIS AND CIRRHOSIS.

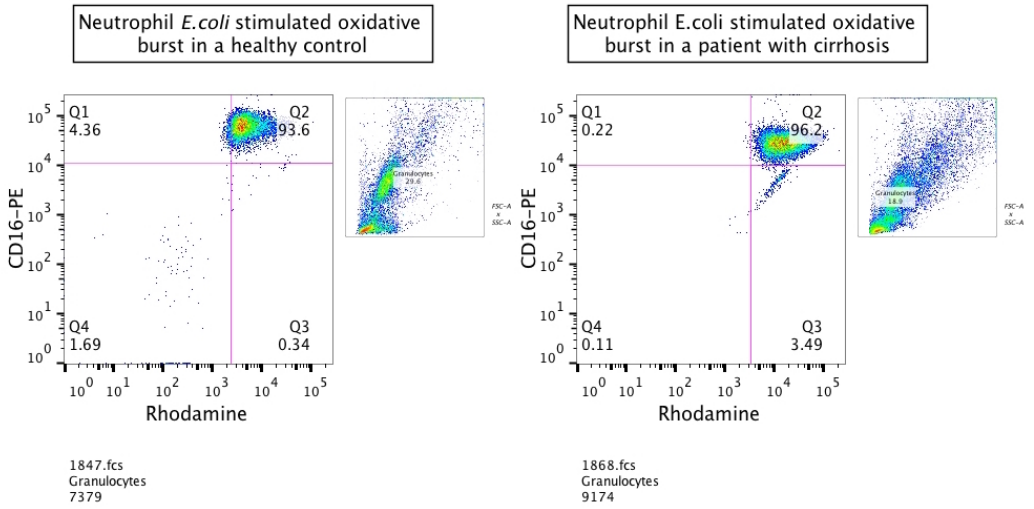


FIGURE 5-9 BURST IN NEUTROPHILS EXPOSED TO OPSONISED *E.COLI* WITH OXIDATION DETECTED BY THE INTENSITY OF RHODAMINE-123 FLUORESCENCE IN A HEALTHY CONTROL AND A PATIENT WITH ACUTE ALCOHOLIC HEPATITIS AND CIRRHOSIS.

5.11 Cytokine estimation using enzyme-.linked immunosorbance assay (ELISA)

Plasma levels of the pro- and anti-inflammatory cytokines (TNFα, IL-1ß, IL-6, CXC8/IL-8, IL-10 and IL-17) were determined from samples previously stored at -80 ºC using sandwich enzyme-linked immunosorbent assay (R&D Systems DuoSets®, UK) and results correlated with neutrophil function and cell volume.

5.11.1 Solutions

A. Capture antibody

- Human TNFα capture antibody - 720 μg/mL of mouse antihuman TNFα when reconstituted with 0.5 mL of phosphate buffered saline (PBS). Diluted to a working concentration of 4.0 μg/mL in PBS, without carrier protein (i.e. 55.6 μL of reconstituted solution added to 9944.4 μL of PBS).
- Human IL-1β capture antibody – 720 μg/mL of mouse antihuman IL-1β when reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 4.0 μg/mL in PBS, without carrier protein (i.e. 55.6 μL of reconstituted solution added to 9944.4 μL of PBS).
- Human IL-6 capture antibody – 240 μg/mL of mouse antihuman IL-1β when reconstituted with 0.5 mL of PBS. Diluted to a working concentration of 2.0 μg/mL in PBS, without carrier protein (i.e. 41.7 μL of reconstituted solution added to 9958.3 μL of PBS).
- Human CXCL8/IL-8 capture antibody - 720 μg/mL of mouse antihuman IL-8 when reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 4.0 μg/mL in PBS, without carrier protein (i.e. 55.6 μL of reconstituted solution added to 9944.4 μL of PBS).
- Human IL-10 capture antibody –360 μg/mL of mouse antihuman IL-10 when reconstituted with 0.5 mL of PBS. Diluted to a working concentration of 2.0 μg/mL in PBS, without carrier protein (i.e. 55.6 μL of reconstituted solution added to 9944.4 μL of PBS).
- Human IL-17 capture antibody - 720 μg/mL of mouse antihuman IL-17 when reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 4.0

μg/mL in PBS, without carrier protein (i.e. 55.6 μL of reconstituted solution added to 9944.4 μL of PBS).

B. Detection antibody

- Human TNFα detection antibody - 45 μg/mL of biotinylated goat antihuman TNFα when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 250 ng/mL in reagent diluent (i.e. 55.6 mL of reconstituted solution added to 9944.4 mL).
- Human IL-1β detection antibody – 54 μg/mL of biotinylated goat antihuman IL-1β when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 300 ng/mL in reagent diluent (i.e. 55.6 mL of reconstituted solution added to 9944.4 mL).
- Human IL-6 detection antibody – 3.0 μg/mL of biotinylated goat antihuman IL-6 when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 50 ng/mL in reagent diluent (i.e. 50 mL of reconstituted solution added to 9950 mL).
- Human CXCL8/IL-8 detection antibody – 3.6 μg/mL of biotinylated goat antihuman IL-8 when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 300 ng/mL in reagent diluent (i.e. 55.6 mL of reconstituted solution added to 9944.4 mL).
- Human IL-10 detection antibody - 54 μg/mL of biotinylated goat antihuman IL-10 when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 300 ng/mL in reagent diluent (i.e. 55.6 mL of reconstituted solution added to 9944.4 mL).
- Human IL-17 detection antibody – 27 μg/mL of biotinylated goat antihuman IL-17 when reconstituted with 1.0 mL of reagent diluent (see below). Diluted to a working concentration of 150 ng/mL in reagent diluent (i.e. 55.6 mL of reconstituted solution added to 9944.4 mL).

C. Standards

- Human TNFα standard – 290 ng/mL of recombinant human TNFα when reconstituted with 0.5 mL of reagent diluent. Diluted to a high standard of 1000

pg/mL (i.e. dilute 3.4 mL of reconstituted solution with 996.6 mL reagent diluent) An eight-point serial dilution was then performed (Figure 5-10).

- Human IL-1β standard – 100 ng/mL of recombinant human IL-1β when reconstituted with 0.5 mL of deionised water. Diluted to a high standard of 500 pg/mL (i.e. 5 mL of the reconstituted solution added to 995 mL reagent diluent). An eight-point serial dilution was then performed.
- Human IL-6 standard - 120 ng/mL of IL-6 when reconstituted with 0.5 mL of reagent diluent. Diluted to a high standard of 1400 pg/mL (i.e. dilute 11.7 mL of reconstituted solution with 988.3 mL reagent diluent). An eight-point serial dilution was then performed.
- Human CXCL8/IL-8 standard – 140 ng/mL of IL-8 when reconstituted with 0.5 mL of reagent diluent. Diluted to a high standard of 750 pg/mL (i.e. dilute 21.4 mL of reconstituted solution with 978.6 mL reagent diluent). An eight-point serial dilution was then performed.
- Human IL-10 standard - 210 ng/mL of IL-10 when reconstituted with 0.5 mL of reagent diluent. Diluted to a high standard of 4200 pg/mL (i.e. dilute 20 mL of reconstituted solution with 980 mL reagent diluent). An eight-point serial dilution was then performed.
- Human IL-17 standard – 220 ng/mL of recombinant human IL-17 when reconstituted with 0.5 mL of reagent diluent. Diluted to a high standard of 2200 pg/mL (i.e. dilute 10mL of reconstituted solution with 990 mL reagent diluent). An eight-point serial dilution was then performed.

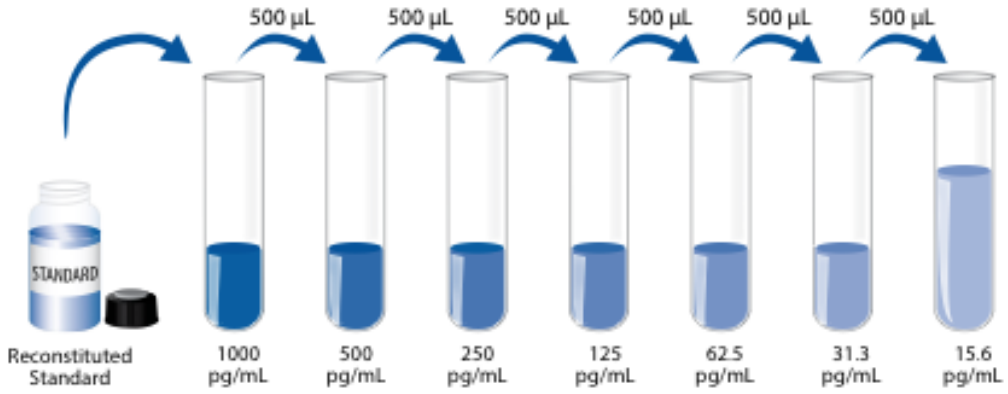


FIGURE 5-10 CARTOON SHOWING HOW A SERIAL DILUTION OF A STANDARD WAS PERFORMED

D. Streptavidin-Horseradish peroxidase (HRP) – 1.0mL of streptavidin conjugated to horseradish peroxidase. Dilute to the working concentration specified.

E. Phosphate Buffered saline – 137mM NaCl, 2.7 mM KCl, 8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, PH 7.2-7.4, 0.2 μM filtered. 5 x PBS tablets were added to 1000 mLs denatured water.

F. Wash buffer – 0.05% Tween® 20 in PBS, pH 7.2-7.4. 5 x PBS tablets were added to 9950 mL of denatured water and 50 μL of Tween® 20

G. Block buffer (IL-8 ELISA only) – 1% BSA, in PBS with 0.05% NaN_3.

H. Reagent Diluent* (100 mLs) – 1% bovine serum albumin (BSA), pH 7.2-7.4, 0.2μM filtered. One gram bovine serum albumin was added to 100 mL PBS from stock.
*The IL-8 ELISA required a different reagent diluent of 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizam base, 150mM NaCl), pH 7.2-7.4, 0.2 μM filtered.

I. Substrate solution – 25 mLs citrate phosphate buffer and 1 x 0-Phenylenediamine dihydrochloride (SIGMAFAST™ OPD) tablet (Sigma-Aldrich)

J. Stop solution - 2 N sulphuric acid (H_2SO_4)

5.11.2 ELISA Plate preparation

1. The capture antibody was diluted to the working concentration in PBS without carrier protein. A 96-well flat-bottomed microplate (R&D systems) was then immediately coated with 100 μL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature.
2. Each well was then aspirated and washed with Wash buffer, repeating the process two times for a total of 3 washes. The wash was performed by filling each well with Wash buffer (440 μL) using a multi-tipped pipette or autowasher. Complete removal of liquid was ensured at each step. After the final wash, remove any

remaining water was removed by inverting the plate and blotting against clean paper towels.

3. The plates were blocked by adding 300 mL of reagent diluent to each well. * Note the IL-8 assay uses a specific block buffer. The plates were then incubated at room temperature for 1 hour.

5.11.3 ELISA assay procedure

1. Add 100 μL of sample or standards were added to reagent diluent or an appropriate diluent (*see IL-8 reagent diluent), per well. Note all samples were performed in duplicate. Samples were covered with an adhesive strip and incubated for 2 hours at room temperature on a rotating microplate mixer
2. The Aspiration/wash steps were repeated as in step 2 of plate preparation.
3. 100 μL of the working concentration of the detection antibody were added to each well. A new adhesive strip was placed to cover and the plate was again incubated for 2 hours at room temperature on a rotating microplate mixer.
4. The Aspiration/wash steps were repeated as in step 2 of plate preparation.
5. 100 μL of the working dilution of Streptavidin-HRP was added to each well. The plate was covered and incubated for 20 min at room temperature. Care was taken to avoid placing the plate in direct light.
6. The Aspiration/wash steps were repeated as in step 2 of plate preparation.
7. 100 μL of substrate solution was added to each well and the plate was incubated for 20 min at room temperature. Care was taken to avoid placing the plate in direct light.
8. 50 μL of Stop solution was added to each well and the plate gently tapped to ensure thorough mixing [Figure 5-11 shows the typical appearance of plates after the addition of stop solution prior to determination of optical density].
9. The Optical density of each well was determined immediately, using a microplate reader set to 492 nm.

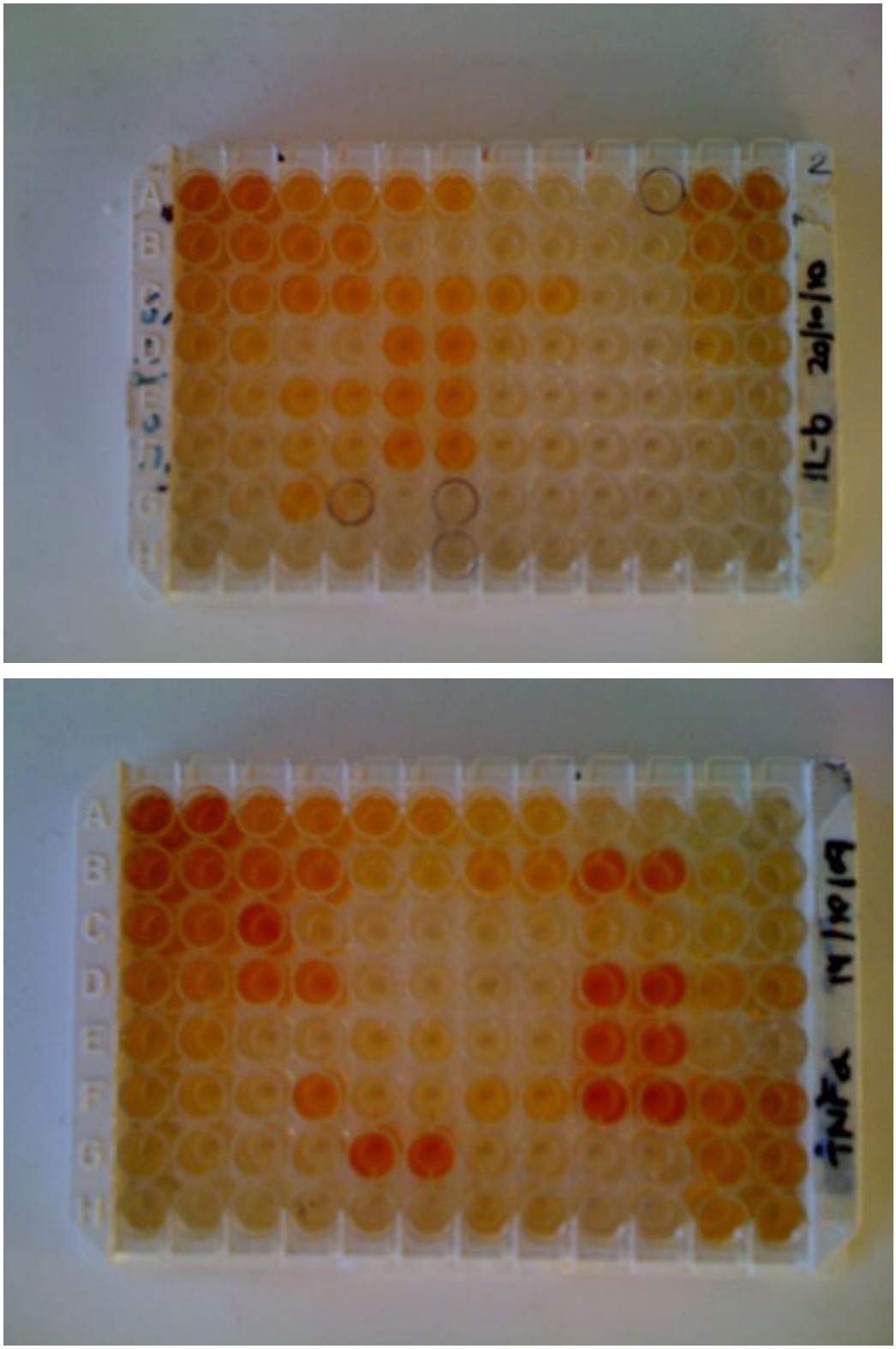


FIGURE 5-11 ELISA ANALYSIS OF A - INTERLEUKIN-6 AND B - TNFα WITH DUPLICATE STANDARDS IN LANES 1 AND 2 FOLLOWED BY 40 DUPLICATE SAMPLES RUNNING IN ADJACENT WELLS.

5.12 Experiment to determine the effects of ammonia, and the p38 agonist Isoproterenol and antagonist SB203580 on neutrophil function and levels of phosphorylated p38-MAPK.

5.12.1 Solutions:

A - Phosphate buffered saline

B – 1M NH_4Cl (MW 54.4). 1M solution = 2.67g NH_4Cl in 50mL double distilled water.

C – 1mM Isoproterenol (MW=247.7). 1mM solution = 0.0124 g isoproterenol in 50mL double distilled water.

D- SB203580 (MW = 377.4). Diluted as per manufacturers instruction in dimethyl-sulphoxide (DMSO) 10 µM = 2 µL of 1 mg vial in 2 mL blood.

5.12.2 Method:

- Two mL of whole blood from control/patient were incubated and mixed with the appropriate solutions according to the schedule in table 5-11 for 90 minutes (mixing every 30 minutes) in a water bath at 37°C as per the above schedule [Table 5-11].
- The Phagotest™ and PhagoBurst™ test were performed as per the above method for the preparation of blood samples for the Phagotest™/PhagoBurst™ test except that 100 µL of the above prepared blood was used instead of the heparinised blood.
- Concentration curves were initially constructed for ammonia, isoproterenol and SB203580 using the following concentrations:
    - Ammonia 100 µM, 200 µM, 400 µM, 800 µM and 1600 µM
    - Isoproterenol 1 nM, 2 nM, 4 nM, 8 nM and 16 nM
    - SB203580 10 µM, 20 µM, 40 µM, 80 µM and 160 µM.

After the construction of concentration curves the following concentrations were selected; ammonia 200 µM, isoproterenol 1 nM and SB203580 10 µM. The effects of changes in extracellular pH and partial pressure of CO2 were assessed along with neutrophil viability using the following methods.

5.12.3 The effect of ammonia on extracellular pH

The pH and partial pressure of carbon dioxide (pCO_2) were measured using a Radiometer ABL 700 Series blood gas analyzer on whole blood samples with 0 - 400 μM NH_4Cl, to assess for changes in extracellular pH resulting from incubating whole blood, and thus the neutrophils, with ammonia. There were no detectable differences in either extracellular pH or pCO2 between controls and whole blood incubated with up to 400 μM NH_4Cl from which the neutrophils were isolated 90 minutes later.

5.12.4 Assessment of neutrophil viability was performed using the following 2 methods:

-Trypan Blue Exclusion Test

There was no difference in the number of non-viable cells between neutrophils isolated from control whole blood and neutrophils isolated from whole blood incubated for 90 minutes with 100 or 400 μM NH_4Cl [1.57, 1.78 and 1.77% non-viable for 0, 100 and 400 μM NH_4Cl, respectively].

-Annexin V-FITC and Propidium Iodide

Two experiments were performed. The first assessed apoptosis/cell death and the duration of incubation with ammonia, and the second assessed the relationship between concentration of ammonia and apoptosis/cell death.

Ammonia-induced apoptosis or necrosis was excluded by staining with propidium iodide and annexin V-FITC. Up to 5 hours of incubation in a waterbath at 37 ºC with 100 μM NH_4Cl resulted in only 1-2% of neutrophils showing evidence of early apoptosis (annexin V-FITC positive, propidium iodide negative) and necrosis/cell death (annexin V-FITC positive and propidium iodide positive) as measured with flow cytometry. After 5 hours, the neutrophils began to die with >20% of them staining positive with both annexin V-FITC and propidium iodide as measured with flow cytometry. Furthermore, incubation with ammonia for up to 120 minutes at concentrations of NH_4Cl up to 400 μM did not induce apoptosis or cell death and this cannot therefore account for the impairment in phagocytosis or increase in spontaneous respiratory burst activity.

Table 5-11 Schedule for treating control and patient blood samples in order to determine the effects of ammonia and p38 modulators on neutrophil function and levels of total and phosphorylated p38-MAPK

Sample	Whole blood	PBS	1M NH_4Cl	1mM Isoprotorenol	SB203580
Control	0	2 mL	0	0	0
Ammonia 200μM	1096 μL	0	4 μL	0	0
Isoproterenol 1nM	1098 μL	0	0	2 μL	0
SB203580 10μM	1098 μL	0	0	0	2 μL
Ammonia 200 μM & Isoproterenol 1 nM	1096 μL	0	2 μL	2 μL	0
Ammonia 200μM & SB203580 10μM	1096 μL	0	2 μL	0	2 μL

*Patients studied had an arterial ammonia concentration of >100μM/L to ensure that they have been exposed to a supra-physiological concentration *in vivo* so that ammonia-induced changes could be determined.

5.13 Experiment to determine total and phosphorylated p38-MAPK using cytometric bead array

5.13.1 Preparation of Cells in Suspension:

1. Cells were treated as per the schedule in Table 5-11 to induce or inhibit p38-MAPK phosphorylation.

2. Cells were counted in the sample (trypan blue exclusion test) and an approximation of the protein concentration (should be >1 mg/ml see protein estimation later on) was calculated.
3. The sample was denature the sample by adding 1 mL ice-cold FACS lysis buffer containing 0.1% Triton ® X-100 (detergent) to the cells. The sample was incubated for 15 minutes at 4°C and pelleted insoluble material was obtained by centrifugation (14,000 rpm for 3 minutes).
4. The supernatant was transferred to a clean tube and add the appropriate amount of 5x denaturation buffer added so that the final concentration of denaturation buffer was 1x.
5. The sample was denatured by immediately placing it in a boiling water bath (or heating block at 100°C) for 5 minutes. The sample may very viscous and difficult to pipette due to the presence of deoxyribonucleic acid [DNA] in which case 40µL of DNase I was added or the sample passed through a 26 gauge needle several times, or both).
6. The protein concentration was determined using the Bradford Reagent*
7. At this point the sample could be stored at -70 °C for up to 6 months, then thawed and centrifuged at 14,000 rpm for 3 min before the sample could be used to pellet debris.
8. The cell lysate sample was diluted to the desired dilutional factor (i.e. 1:2, 1:10 or 1:20) using the appropriate volume of assay diluent. It was vital that the sample must be diluted at least 1:4 to reduce percentage of sodium dodecyl sulphate (SDS) and the lysate should not contain more than 20µg total protein.
9. Sample dilutions were mixed thoroughly before transferring to the appropriate assay tubes containing capture beads.

5.13.2 Preparation of cytokine bead array cell signalling flex set standards

1. The lyophilised sphere was transferred to a 1.5 mL microfuge tube.
2. The standard was reconstituted adding 100 µL of assay diluent, warming the tube to 37°C and vortexing.

3. Once reconstituted, the standard was stored at 4°C and was stable for 3 months. If using a frozen reconstitute standard, it was re-warmed and vortexed thoroughly.
4. Falcon tubes were labelled and arranged in the following order:
    a. Top standard: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 (2 further dilutions could be performed if low levels expected).
5. 20 µL of each Cell Signalling BD CBA Flex Set Standard to be run in the experiment were added to the Top Standard tube.
6. Assay Diluent (yellow buffer) was added to the Top Standard Tube to bring the volume up to 1mL.
7. 500 µL of Assay Diluent were added to each of the remaining tubes.
8. A serial dilution was performed [Figure 5-12].
9. Assay diluent alone served as a negative control.
10. Standards were run in the order of the least concentrated (assay diluent) to the most concentrated (Top standard).

5.13.3 Preparation of cytokine bead array cell signalling flex set capture beads

The Capture beads are at a 50x concentration and must be diluted to their optimal concentration before adding to a given assay tube or well.

- The number of flex sets to be used was determined (size of multiplex, in our case two).
- The number of tests per experiment was determined. (A few additional tests were prepared so as not to run out).
- Each capture bead was vortexed thoroughly for 15 seconds.
- The total volume to be used was determined by multiplying the number of tests by 50 µL.
- The volume of each capture bead (1 µL = 1 test) was determined so the required volume in µL was equal to the number of tests.
- The volume of Capture Bead Diluent needed to dilute the beads was determined by subtracting the volume of capture bead from the total volume required.

- The Capture Beads and Capture Bead Diluent were pipetted into a tube labelled Mixed Capture Beads.

5.13.4 Preparation of cytokine bead array cell signalling flex set PE Detection Reagents

The PE detection reagent is at a 50x concentration and was diluted to the optimal concentration before adding to a given assay tube or well. This section could be performed during the initial 3-hour incubation of capture beads with samples/standards.

- Determination of the number of flex sets to be used (size of multiplex). The number of tests per experiment was determined. It was recommended that the user prepare a few additional tests than they anticipate using to ensure that there was enough material prepared for the experiment.
- The total volume to be used was determined by multiplying the number of tests by 50 µL. The PE detection reagent was brought to room temperature and mixed well before use. The solution was somewhat viscous so care was taken in making sure that the exact volume was pipetted.
- The volume of each PE Detection Reagent (1 µL = 1 test) was determined so the required volume in µL was equal to the number of tests.
- The volume of Detection Reagent Diluent needed to dilute the PE detection reagent was determined by subtracting the volume of PE detection reagent from the total volume required.
- The detection reagents and Detection reagent diluent were pipetted into a tube labelled Mixed PE Detection Reagents and stored at 4 °C, protected from light until ready to use.

5.13.5 Cytokine bead array cell signalling flex set assay procedure

- All reagents were prepared as described in previous sections before starting the experiment.
- The mixed Capture Beads were vortexed for at least 5 seconds and 50 µL of the mixed capture beads were added to each assay tube.
- 50 µL of Standard or sample were added to the assay tubes.

- The assay tubes were gently mixed and incubated for 3 hours at room temperature whilst being protected from direct exposure to light.
- 50 µL of the Mixed PE Detection Reagent was added to each assay tube. The tubes were gently mixed and incubated for 1 hour at room temperature and protected from direct sunlight (note once Mixed PE Detection Reagent is added, the liquid in each well should appear green in colour).
- One µL of Wash Buffer was added to each assay tube and centrifuged at 1600 rpm for 5 minutes.
- The supernatant was carefully aspirated and discarded from each assay tube.
- 300 µL of Wash Buffer was added to each assay tube which were briefly vortexed to re-suspend beads.
- The samples were then analysed on a flow cytometer. It was recommended that each tube be mixed gently before analysing on the FACS. The appropriate FACS instrument instruction manual was referred to for acquiring the BD CBA Flex Sets.

5.13.6 Bradford assay

This method involves the binding of the Coomassie Brilliant Blue to the proteins in acidic medium. The binding of the dye to protein causes a shift in the absorption maxima from 465nm to 595nm. This shift is observed and measured at 595nm using a spectrophotometer. (Bradford, 1976) This is a rapid and stable colorimetric assay. Increasing protein concentration increases the presence of bound dye, hence a concentration dependent colorimetric response can be obtained.

1. The reagent was brought to room temperature and gently vortexed.
2. The protein standards were then prepared, ranging from 0.1-1.4 mg/mL [Table 5-11].
3. Five µL of the standards were added to each well on a 96-well plate in duplicate. The blank control was made by adding 5 µL of Radioimmunoprecipitation assay (RIPA) buffer.
4. The samples whose protein concentration was being determined was then added in duplicate, assuming the range of the unknown to be in between 0.1-1.4 mg/mL.

5. 250 µL of the Bradford reagent was then added to each well and mixed for 30 seconds.
6. This was then incubated at room temperature for 30 minutes [Figure 5-12 illustrates a typical plate appearance after incubation].
7. The absorbance at 595nm was then read in a UV spectrophotometer.
8. Absorbance versus protein concentration for each standard was then plotted. Unknown concentrations of protein in the samples were determined by plotting against the standard curve.

Table 5-12 Serial dilution of protein standard for Bradford assay to determine protein concentration in a solution

STANDARD SAMPLE BSA (mg/mL)	VOLUME OF BSA STOCK (µL)	VOLUME OF WATER (µL)
0	0	1000
0.1	100	900
0.2	200	800
0.4	400	600
0.6	600	400
0.8	800	200
1	1000	0

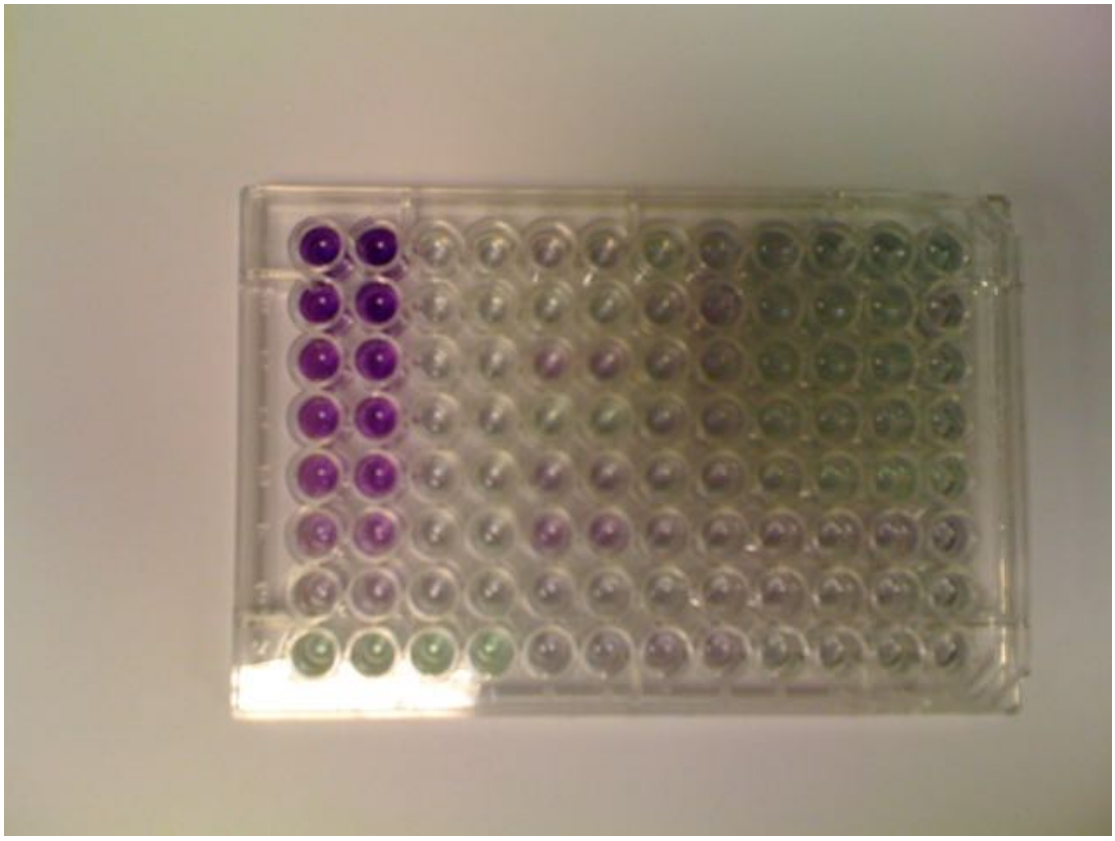


FIGURE 5-12 BRADFORD ASSAY PLATE WITH DUPLICATE PROTEIN STANDARDS IN LANES 1 AND 2 RUNNING FROM HIGHEST PROTEIN CONCENTRATION IN THE WELLS AT THE TOP LEFT-HAND CORNER. DUPLICATE PATIENT SAMPLES WERE RUN IN ADJACENT WELLS

5.13 Statistics

Where appropriate, values are expressed as median and inter-quartile range. Group comparisons were performed using the Chi-squared test for categorical and Mann-Whitney *U* test for continuous variables. Paired-sample comparisons were performed using the Wilcoxon matched-pair test for categorical variables. Correlation between 2 continuous variables was performed using linear regression. $P<0.05$ was considered as statistically significant. Non-parametric Area Under the Receiver Operator Curve (AUROC) curve analysis was performed for outcome measures. Survival analysis was performed using the Kaplan-Meier log-rank method. Statistical analyses were performed using GraphPad Prism 4.0 for Mac (GraphPad Software, Inc., San Diego, CA). Multivariate analysis was performed using SPSS 16.0 for Macintosh (SPSS Inc, CA, 2007.) Image-J software (ImageJ.nih.gov) was used for transmission electron micrograph analysis.

Chapter 6 – Circulating neutrophil dysfunction in acute liver failure

6.1 Introduction

Acute liver failure (ALF) is a rare but frequently catastrophic consequence of an acute primary hepatic injury arising from a wide variety of insults. It is characterised by coagulopathy and encephalopathy, with a variable dynamic of progression to multiple organ dysfunction syndrome (MODS) and death. (Trey and Davidson 1970) Liver transplantation (LT) remains the only curative option in advanced ALF with poor prognostic criteria and contributes to 10% of liver transplants in the western world. (Bernal et al., 2010)

Neutrophils are rapidly recruited to the liver in response to hepatic injury in ALF, (Schlayer et al., 1988); once there they become activated by cytokines (e.g. IL-8 and TNFα) and can contribute to tissue damage by release of proteolytic enzymes and reactive oxygen species (ROS). (Ramaiah and Jaeschke, 2007) Systemic neutrophil activation with associated immuneparesis is well recognised in severe sepsis; a condition which shares many phenotypic features with ALF including microvascular dysfunction, haemodynamic instability, coagulopathy, encephalopathy and high levels of both pro-inflammatory and anti-inflammatory cytokines. (Brown et al., 2006) In severe sepsis excessive activation of neutrophils, due to high circulating levels of cytokines and Toll-like receptor activation by endotoxin (LPS), has been implicated in the pathogenesis of acute lung and kidney injury (Awad et al., 2009). Neutrophils might therefore serve as critical effector cells of the progressive parenchymal liver damage and MODS in ALF.

There is a high incidence of bacterial and fungal infection early in the course of ALF (Karvellas et al., 2009) which may preclude listing for LT. ALF is also associated with an acute and often precipitous increase in plasma ammonia levels. (Bernal et al., 2010) A recent study has shown that neutrophils exposed to ammonia have reduced phagocytic activity of opsonised *E. coli* and high spontaneous production of ROS suggesting a direct toxic effect of ammonia on neutrophils. (Shawcross et al., 2008a) Neutrophil dysfunction has also been previously reported in ALF with reduced complement expression, (Wyke et al., 1982) impaired neutrophil adhesion,(Altin et al., 1983) decreased production of ROS (Clapperton et al., 1997), and decreased neutrophil phagocytosis and intracellular killing (Rolando et al., 2000a).

I postulate that circulating neutrophil dysfunction is present in ALF and may add value as a prognostic marker of severity and outcome. The aim of this prospective case-control longitudinal study was therefore to characterise circulating neutrophil phenotype,

phagocytic activity (NPA) and production of ROS, in neutrophils isolated from the peripheral blood of patients with ALF and comparing it to healthy (HC) and septic controls (SC). Indices of neutrophil phenotype and function were examined in respect of severity and nature of liver injury, severity of organ failure, liver prognostic criteria of survival and eventual outcome. The relationship between plasma-derived factors and neutrophil function was also examined in order to aid identification of other associated biomarkers in ALF.

6.2 Patients recruited

Twenty-five patients with ALF or SALF were recruited non-consecutively on admission to the liver ICU at King's College Hospital between October 2008 and August 2010. ALF was defined by the onset of hepatocellular dysfunction in the absence of pre-existing liver disease characterised by coagulopathy and encephalopathy and an illness of less than 26 weeks duration. ALF was further sub-classified according to the criteria defined by O'Grady et al. (O'Grady et al., 1993) depending on the time between the onset of jaundice and encephalopathy.

(i) **Hyperacute** (jaundice to encephalopathy time <7 days) consisting predominantly of patients with acetaminophen-induced liver failure (AALF).

(ii) **Acute liver failure** (jaundice to encephalopathy time 8-28 days) typified by patients presenting with fulminant viral hepatitis.

(iii) **Sub-acute Liver Failure** (SALF) (jaundice to encephalopathy time 5-12 weeks) typified by those presenting with non-acetaminophen drug-induced liver injury and seronegative/acute autoimmune hepatitis.

6.2.1 Inclusion Criteria

Patients with ALF/SALF were included if they were aged >18 years and <80 years. Healthy age and sex-matched non-smoking volunteers with no history of liver disease were used as HC. The HC alcohol intake was <20 g/day and volunteers had not drunk alcohol or exercised excessively in the 24 hours prior to blood being drawn. SC patients were recruited from the general ICU and had severe sepsis with MODS. Severe sepsis was defined by the presence of a SIRS score ≥ 2 (16), with radiological and/or laboratory evidence of infection and 1 or more extra-hepatic organ failure(s). Patients presenting

with ALF/SALF were given empirical intravenous antibiotic and antifungal cover as standard of care. This consisted of tazocin 4.5 grams every 8 hours (substituted for meropenem 1 gram every 8 hours if penicillin allergic) and fluconazole 400mg once daily.

6.2.2 Exclusion criteria

Patients were excluded from the ALF/SALF cohorts if on presentation they had evidence of bacterial, fungal or viral infection on clinical examination, radiological or laboratory investigation, malignancy and any coexisting history of immunodeficiency including HIV and glycogen storage disease. Patients with pre-existing liver disease, a history of alcohol intake >20g/day or who were on immunosuppressive therapies such as steroids or azathioprine were also excluded.

6.3 Patient baseline demographics and clinical parameters

15 non-consecutive patients with ALF and 10 patients with SALF were recruited. Baseline (on admission to ICU) patient demographics, biochemical and physiological parameters are detailed in Tables 6-1, 6-2 and 6-3, respectively. The ALF group were heterogeneous in terms of aetiology and severity of liver injury [acetaminophen n=6; acute viral hepatitis n=3; other n=6]. Other aetiologies in the ALF cohort included one each of seronegative autoimmune hepatitis, Budd-Chiari syndrome, ischaemic hepatitis and eosinophilic hepatitis, with 2 further patients suffering a drug-induced liver injury (DILI). The predominant aetiology in SALF was seronegative / acute autoimmune hepatitis n=7, other aetiologies included hepatitis B virus infection in one patient and DILI in a further two patients.

Within the ALF (n=15) cohort 9 (60%) fulfilled King's College Hospital criteria for poor prognosis, (O'Grady et al., 1989) of whom 4 (44%) underwent successful LT, 4 (44%) were declined LT due to co-morbidity and 1 (12%) was listed but died of cerebral oedema before a graft became available. One patient met poor prognostic criteria but was declined LT due to psychiatric co-morbidity but survived following plasmapheresis. In the SALF (n=10) cohort 8 (80%) fulfilled poor prognostic criteria; of whom 6 (60%) underwent LT; 1 (10%) was declined due to co-morbidity; and 1 (10%) recovered and was de-listed. Two SALF patients died (one post-LT from MODS).

All patients with ALF/SALF were significantly unwell with MODS and indeed, MELD and SOFA scores were significantly higher in the ALF and SALF cohorts compared to the SC ($p=0.001$ and $p=0.0035$, respectively) [Table 6-2]. Patients with SALF had a tendency to be older, with higher bilirubin and lower arterial ammonia, but due to the small numbers in the groups, these comparisons did not reach significance.

For underlying diagnosis in the septic controls see Table 6-1. Co-morbidities included chronic obstructive pulmonary disease in the faecal peritonitis patient and previous stroke, hypertension, diabetes mellitus and hypercholesterolaemia in the pneumonia patient.

Table 6-1 Baseline demographic data and outcomes for patients with ALF/SALF

	Severe sepsis	Acute Liver Failure	Sub-acute Liver Failure	Liver Failure Spontaneous Survivors	Liver Failure Death / LT
Number	6	15	10	10	15
Median Age	40.5 (24-70)	33 (26-48)	52.5 (44-60)	34 (28-44)	51 (32-59)
Female	2 (33)	10 (67)	6 (60)	7 (70)	9 (60)
Aetiology					
Acetaminophen		6 (40)	-	3 (30)	3 (20)
Viral hepatitis		3 (20)	1 (10)	2 (20)	2 (13)
Autoimmune hepatitis		1 (7)	7 (70)	3 (30)	5 (33)
Drug-induced (non-acetaminophen)		2 (13)	2 (20)	-	4 (27)
Other ALF		3 (20)	-	2 (20)	1 (7)
Aetiology of Sepsis					
Pancreatic pseudocyst	3 (50)				
Aspiration pneumonia	1 (17)				
Infective endocarditis	1 (17)				
Faecal Peritonitis	1 (17)				
Transplant-free 90-day survival		7/15 (40)	3/10 (30)	10/10 (100)	0 (0)
Poor prognostic criteria*		9/15 (60)	8/10 (80)	2/10 (20)	15 (100)
Declined LT		4/9 (44)	1/10 (10)	2/2 (100)	3/15 (20)
Underwent LT		4/9 (44)	6/10 (60)		10/15 (67)
Listed for LT but died before grafted		1/9 (12)	-		1/15 (7)
Listed but survived without LT		-	1/10 (10)		1/10 (10)
Died (%)	4 (67)	4/15 (27)	2/10 (20)		6/15 (40)

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate.
* Fulfilled King's College Hospital poor prognostic Criteria.
Abbreviations: ALF- Acute Liver failure; SALF – Sub-acute Liver Failure; HAV-Hepatitis A virus; HBV-Hepatitis B virus; HEV-Hepatitis E virus; LT-Liver transplantation.

Table 6-2 Clinical and organ failure parameters of patients with ALF/SALF on day of recruitment.

Clinical/Biochemical/Organ Failure Variables	Severe sepsis n=6	ALF n=15	SALF n=10	p-value (Kruskal Wallis)
Body Mass Index	24.5 (20.8-29.7)	21.8 (21.4-24.7)	25.3 (23.2-27.4)	0.74
Antibiotics and antifungals*	6 (100%)	13 (87%)	9 (90%)	0.65
Grade of encephalopathy				0.62
Grade 0-2		4 (27%)	5 (50%)	
Grade 3-4		11 (73%)	5 (50%)	
Mean arterial pressure (mmHg)	83 (74-88)	71.5 (67-81.5)	80 (73-94)	0.05
Number requiring invasive ventilation	3 (50%)	9 (60%)	6 (60%)	0.91
Number requiring vasopressors	4 (66%)	6 (40%)	3 (30%)	0.36
Number requiring hemofiltration	2 (33%)	10 (66%)	6 (60%)	0.37
Days on hemofiltration	3 (3-5)	4.0 (3.25-7.25)	4.0 (1.5-5.0)	0.63
Organ failure scores				
SIRS Score	3 (2-3)	2 (1-2.5)	1 (1-2)	0.22
MELD Score	13 (9-14)	34.2 (31.7-41.4)	42.4 (28.1-48.8)	**0.001 Δ**
SOFA Score	6 (4.5-6.5)	16 (14.5-17.0)	16 (15.5-17.5)	**0.04 Δ**
APACHE II Score	14 (11-17)	21 (17.5-24.5)	22 (18.5-23.5)	0.17

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate.
Abbreviations: ALF – Acute Liver Failure; SALF - Sub-acute Liver Failure; MELD-Model of End-stage liver disease; SOFA-Sequential Organ Failure Score; APACHE II-Acute Physiology and Chronic Health Evaluation II. SIRS-Systemic inflammatory response syndrome score

***** Patients presenting with ALF/SALF were given empirical intravenous antibiotic and antifungal cover as standard of care. This was tazocin 4.5 grams every 8 hours (substituted for meropenem 1 gram every 8 hours in those who were penicillin allergic) and fluconazole 400mg once daily.

Δ = Values in both ALF and SALF cohorts were significantly increased compared with septic controls. The Kruskal-Wallis test was utilised with Dunn's multiple comparison test and $p<0.05$ was considered statistically significant.

Table 6-3 Biochemical and neutrophil parameters of patients with ALF/SALF on day of recruitment

Biochemical & Neutrophil Variables	**Severe sepsis N=6**	**Acute liver failure N=15**	**Subacute liver failure N=10**	**p-value (Kruskal Wallis)**
Haemoglobin (g/dL)	9.0 (8.2-9.7)	9.5 (8.2-11)	9.3 (8.7-12.8)	0.44
Neutrophil count (x 10^9^L)	11.7 (8.2-15.5)	7.5 (5.5-11.0)	7.9 (5.1-10.1)	0.31
INR	1.32 (1.19-1.38)	3.21 (2.25.4.70)	3.18 (2.27-7.15)	**0.002 Δ**
Aspartate Aminotransferase (IU/L)	35 (32-42)	386 (237-2577)	203 (131-1062)	**0.001 Δ**
Bilirubin (μmol/L)	9 (7-10)	106 (80.0-210.5)	189 (136.8-266.0)	**0.001 Δ**
Albumin (g/L)	27 (20-29)	21 (19.5-25.0)	17 (13.8-27.0)	0.39
Sodium (mmol/L)	142 (137-146)	142 (140.5-145.5)	143 (136.5-144.8)	0.90
Creatinine (μmol/L)	105 (69-138)	161 (89.5-214.0)	132 (89.3-120.5)	0.51
Lactate (mmol/L)	0.9 (0.7-1.2)	2 (1.4-3.0)	2 (1.65-2.5)	**0.025 Δ**
C-reactive protein (mg/L)	226 (116-349)	17.3 (5.3-31.8)	23 (8.1-29.5)	**0.001 §**
Arterial ammonia (μmol/L)	22 (19-25)	75 (62-107)	61.5 (44-84)	**0.001 Δ**
High Density Lipoprotein (mmol/L)	0.5 (0.35-0.65)	0.35 (0.18-0.50)	0.1 (0.10-1.13)	**0.02 ■**
Arterial pH	7.4 (7.38-7.43)	7.4 (7.37-7.46)	7.49 (4.43-7.52)	0.15

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate.
Δ = Values in both Acute liver failure (ALF) and subacute liver failure (SALF) cohorts were significantly increased compared with septic controls, §= C- reactive protein was significantly greater in septic controls compared with both ALF and SALF cohorts, ■ = High density lipoprotein values were significantly lower in SALF cohort compared to the ALF cohort. The Kruskal-Wallis test was utilised with Dunn's multiple comparison test and $p<0.05$ was considered statistically significant

Table 6-4 Neutrophil function test and cytokine results in acute / subacute liver failure.

	Severe sepsis (N=6)	**Acute liver failure (N=15)**	**Subacute liver failure (N=10)**	**p-value (Kruskal Wallis)**
Neutrophil function				
Phagocytic Activity (%)	70.2 (55.6-78.3)	66.0 (48.8-81.5)	39.6 (32.5-63.9)	0.022
Spontaneous oxidative burst (%)	6.8 (6.3-8.1)	13.8 (6.4-22.8)	6.3 (2.7-10.0)	0.817
E. Coli stimulated oxidative burst (%)	52.2 (39.3-75.3)	85.4 (76.3-92.0)	85.8 (71.8-90.5)	0.079
Plasma cytokines				
Tumour necrosis factor-α (pg/ml)	20 (17-67)	47 (30-114)	69 (15-128)	0.18
Interleukin-1β (pg/mL)	872 (69-2652)	2887 (1236-9053)	7603 (2897-18128)	0.06
Interleukin-6 (pg/mL)	96 (75-172)	140 (51-821)	73 (25-237)	0.47
Interleukin -8 (pg/mL)	65 (34-74)	154 (71-239)	98 (73-304)	0.20
Interleukin -10 (pg/mL)	78 (20-289)	247 (36-896)	299 (55-623)	0.06
Interleukin -17 (pg/mL)	78 (20-289)	227 (72-891)	358 (109-625)	**0.03***

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate. The Kruskal-Wallis test was utilised with Dunn's multiple comparison test and $p<0.05$ was considered statistically significant.
*=Values in both acute liver failure and subacute liver failure cohorts were significantly increased compared to septic controls.

Table 6-5 Neutrophil function test results determined by day of admission to liver unit and post-liver transplant in patients with acute/subacute liver failure.

	Liver failure (day 0-4)	Liver Failure (day 5-10)	Liver Failure (day 10-20)	Post LT
Phagocytic Activity (%)	58.7 (41.2-78.8)	63.9 (45.9-69.3)	72.3 (62.6-85.8)	75.7 (60.0-85.1)
Spontaneous oxidative burst (%)	10.4 (3.3-18.7)	26.4 (7.0-44.5)	6.1 (5.0-9.5)	8.9 (1.2-21.3)
***E. Coli* stimulated oxidative burst (%)**	87.2 (75.1-92.0)	83.0 (74.1-91.2)	72.7 (64.9-87.0)	77.0 (70.5-85.0)

Table 6-6 Clinical and organ failure parameters of spontaneous survivors versus non-survivors/transplanted with ALF/SALF on day of sampling

Clinical/Biochemical/Organ Failure Variables	Liver Failure Spontaneous Survivors (N=10)	Liver Failure Death / LT N=15	p-value
Grade of encephalopathy			0.19
Grade 0-2	6 (60%)	5 (33%)	
Grade 3-4	4 (40%)	10 (67%)	
Mean arterial pressure (mmHg)	77 (70-95)	74 (68.5-79)	0.52
Number requiring invasive ventilation	4 (40%)	11 (73%)	0.10
Number requiring vasopressors	2 (20%)	8 (53%)	0.10
Number requiring hemofiltration	5 (50%)	11 (73%)	0.23
SIRS Score	1 (1-2)	2 (1-2.5)	0.60
Organ Failure scores			
MELD Score	32 (26-42)	42 (33-49)	0.71
SOFA Score	16 (14-16)	16 (15-17)	0.50
APACHE II Score	22 (10-22)	21 (19-26)	0.39

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate.

Abbreviations: LT: liver transplantation: MELD-Model of End-stage liver disease; SOFA-Sequential Organ Failure Score; APACHE II-Acute Physiology and Chronic Health Evaluation II. SIRS-Systemic inflammatory response syndrome score

† Significant differences between spontaneous survivors and death/transplanted are shown using the Mann-Whitney U test; $p<0.05$ was considered statistically significant.

Table 6-7 Biochemical and neutrophil parameters of spontaneous survivors versus non-survivors/transplanted with ALF/SALF.

Clinical/Biochemical/Organ Failure Variables	Liver Failure Spontaneous Survivors (N=10)	Liver Failure Death / LT (N=15)	p-value
Haemoglobin (g/dL)	10.7 (8.1-11.5)	9.3 (8.9-10.9)	0.98
Neutrophil count (x 10^9^L)	5.5 (4.1-6.1)	10.1 (6.8-12.1)	**0.01** †
INR	4.6 (2.9-6.6)	3.3 (.8-5.6)	0.24
Aspartate Aminotransferase (IU/L)	317 (164-1047)	386 (192-1362)	0.57
Bilirubin (μmol/L)	106 (82-125)	204 (134-285)	**0.048** †
Albumin (g/L)	21 (18.5-28.5)	20 (16-26)	0.50
Lactate (mmol/L)	2.1 (1.2-2.8)	1.95 (1.7-2.5)	0.64
Arterial ammonia (μmol/L)	69 (60-94)	144 (91-199)	0.81
High Density Lipoprotein (mmol/L)	0.3 (0.1-0.4)	0.1 (0.1-0.3)	0.35
Arterial pH	7.47 (7.4-7.5)	7.43 (7.38-7.47)	0.22
Neutrophil function			
% Neutrophil Phagocytic Activity	78.2 (56.3-83.3)	53.6 (36.6-69.0)	**0.047** †
% Neutrophil Resting Burst	10.1 (3-18)	10.4 (4-25)	0.77
% Neutrophil Stimulated Burst	85.3 (66-91)	85.8 (76-94)	0.37
Plasma Cytokines			
TNFα pg/mL	85 (25-103)	47 (27-103)	0.64
IL-1 pg/mL	2887 (1277-4212)	7697 (1591-22572)	0.36
IL-6 pg/mL	81 (59-140)	87 (32-559)	0.49
IL-8 pg/mL	129 (47-377)	115 (76-239)	0.25
IL-10 pg/mL	188 (46-411)	479 (57-920)	0.96
IL-17 pg/mL	74 (37-281)	418 (162-926)	0.90

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate.
† Significant differences between spontaneous survivors and death/transplanted are shown using the Mann-Whitney U test; $p<0.05$ was considered statistically significant.

6.4 Neutrophil phenotype

Neutrophil surface receptor expression of CD16 (FcγRIII) and CD11b (Mac-1) was performed on days 1, 4 and 7 in 8/15 of the ALF cohort and compared to HC (n=8) and SC (n=5). Neutrophil expression of CD16 was significantly reduced in the ALF cohort compared to HC ($p<0.001$) on day 1. [Figure 6-1] CD16 expression was also reduced in the SC group compared to HC but this did not reach statistical significance. The CD16 downregulation persisted in the ALF group on days 4 and 7 regardless of outcome but normalized within 72 hours post-LT. No differences were observed in neutrophil surface receptor expression of CD11b in patients with ALF/SALF or in SC (data not shown). Reduced expression of CD62L (L-selectin) was seen in a sample from a 23 year old male with ALF due to eosinophilic hepatitis who was admitted with jaundice, coagulopathy and low-grade encephalopathy. A sample analysed on day 4 of admission, following resolution of HE, showed 26% of his resting neutrophils expressed an activated phenotype with low CD62L expression (normal ≤ 10%).

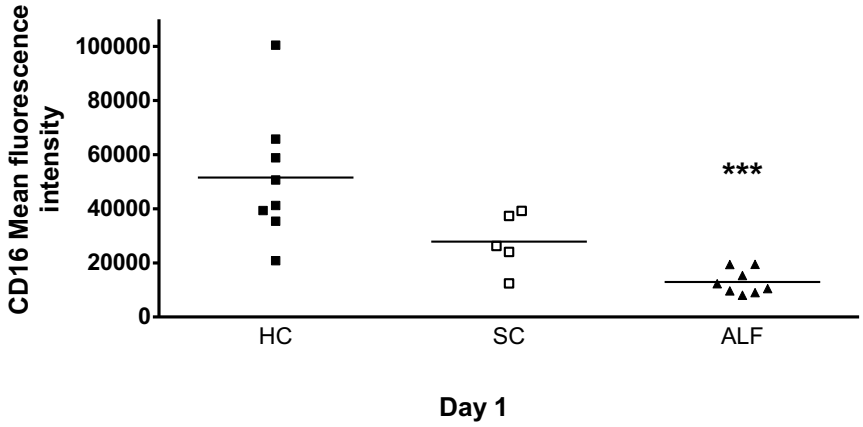


FIGURE 6-1 NEUTROPHIL CD16 EXPRESSION ON DAY 1 OF ICU ADMISSION (IMMUNOGLOBULIN G: FCγRIII) IN HEALTHY CONTROLS (N=8) AND SEPTIC CONTROLS (N=5) COMPARED TO PATIENTS WITH ACUTE LIVER FAILURE [ALF] (N=8)

Differences between healthy controls and patients were calculated using the Kruskal-Wallis test ($p=0.0006$) with Dunn's multiple comparison test. ALF patients had significantly reduced neutrophil CD16 expression compared to controls ($p<0.001$ ***).

6.5 Neutrophil phagocytic activity (NPA)

Neutrophils isolated from the ALF, SALF and SC cohorts on day 1 all demonstrated reduced NPA compared to HC [median (IQR) NPA in the cohorts were as follows: HC 77.7% (72.8-83.7), SC 70.2% (55.6-78.3), ALF 66% (48.8-81.5) and SALF 39.6% (32.5-63.9)] [Table 6-4 and Figure 6-2 and 6-3]. The SALF group showed the greatest reduction in NPA (SALF versus HC $p<0.01$). NPA in the SC cohort showed a non-significant reduction in NPA compared to HC's. Overall, NPA remained depressed on follow up ICU admission days ($p=0.047$) in the ALF/SALF cohorts compared to HC [Table 6-5]. Figure 6-4 charts the typical NPA trend observed on admission and on days 5 and day 9 in an ALF and SALF survivor compared to that observed in an ALF transplanted and SALF death. NPA was significantly improved 72 hours post-LT compared to pre-LT levels; $p=0.03$ [Figure 6-5].

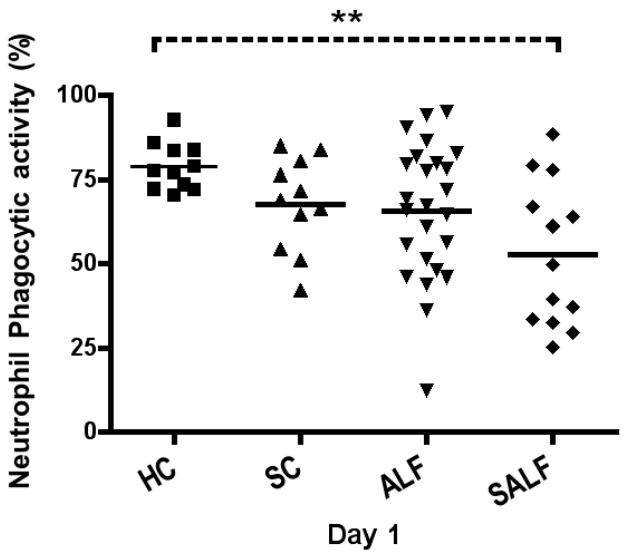


FIGURE 6-2 PERCENTAGE NEUTROPHIL PHAGOCYTIC ACTIVITY OF 11 HEALTHY CONTROLS (HC), 6 SEPTIC CONTROLS (SC), 15 ACUTE LIVER FAILURE (ALF), AND 10 SUBACUTE LIVER FAILURE (SALF) PATIENTS

Differences between HC and patients were calculated using the Kruskal-Wallis test ($p=0.014$) with Dunn's multiple comparison test. Neutrophil phagocytic activity was significantly reduced in the SALF group compared to HC ($p<0.01$ **).

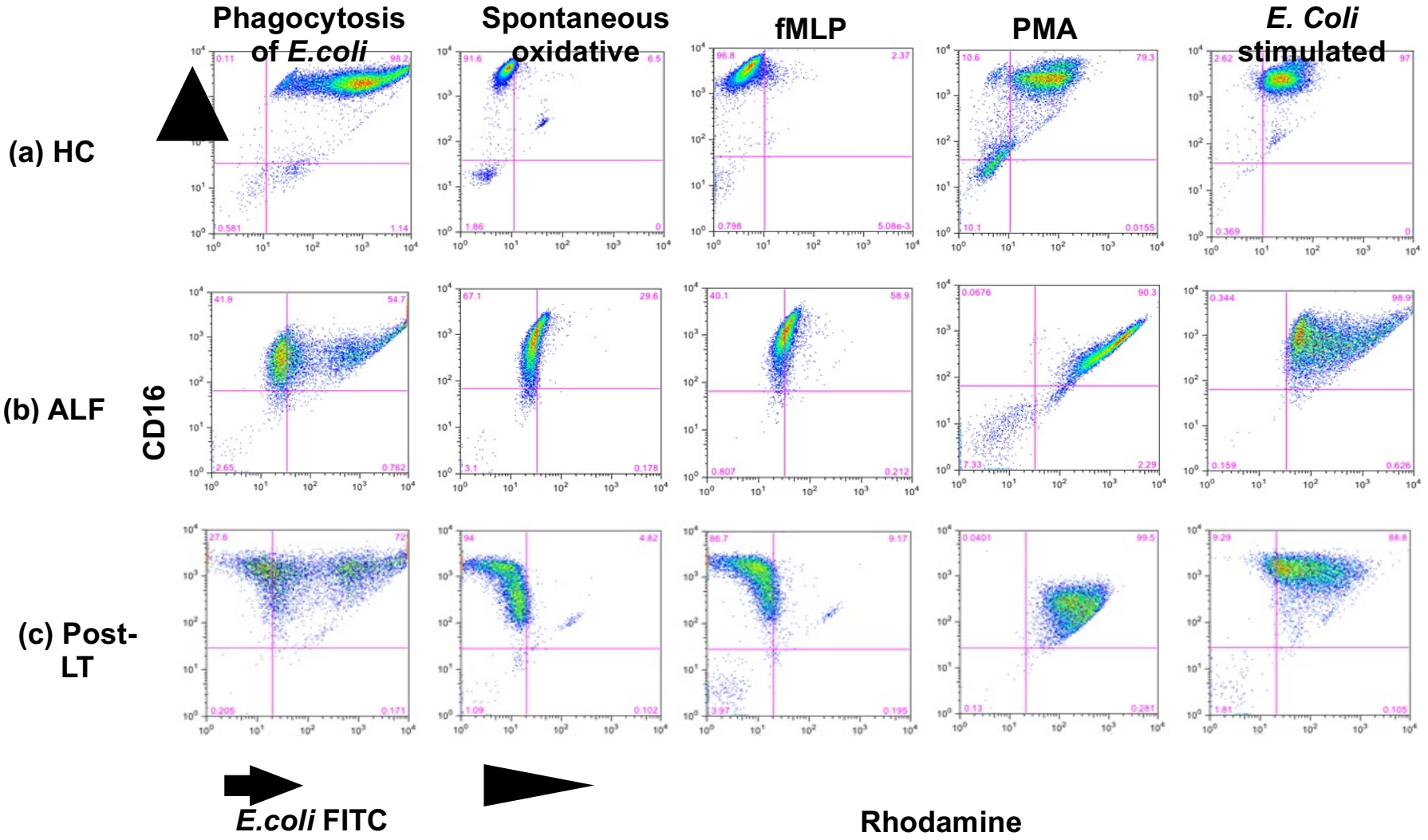


FIGURE 6-3 REPRESENTATIVE FACS PLOTS OF NEUTROPHIL FUNCTION TESTS IN (A) A HEALTHY CONTROL (HC), (B) A PATIENT WITH ACETAMINOPHEN-INDUCED ALF (AALF) AND (C) THE AALF PATIENT 72 HOURS POST-LT.

(i) Shows the plots obtained for phagocytic activity with opsonised *E. coli* labelled with FITC, (ii) Spontaneous (unstimulated) oxidative burst, (iii) Oxidative burst with fMLP, (iv) Oxidative burst with PMA and (v) Oxidative burst with opsonised *E. coli*. The x-axis of the plots represents FITC/rhodamine fluorescence intensity and the y-axis represents CD16 fluorescence intensity. Gates were set using the patients unlabelled (double negative) neutrophils. Neutrophils that have undergone phagocytosis/burst appear in the upper right quadrant of each plot and the overall percentage of neutrophils is labelled. These plots show that in this representative ALF patient there is reduced phagocytic activity compared to a HC (54.7 versus 92.7%) and increased spontaneous oxidative burst (29.6 versus 6.5%) which is normalised post-LT

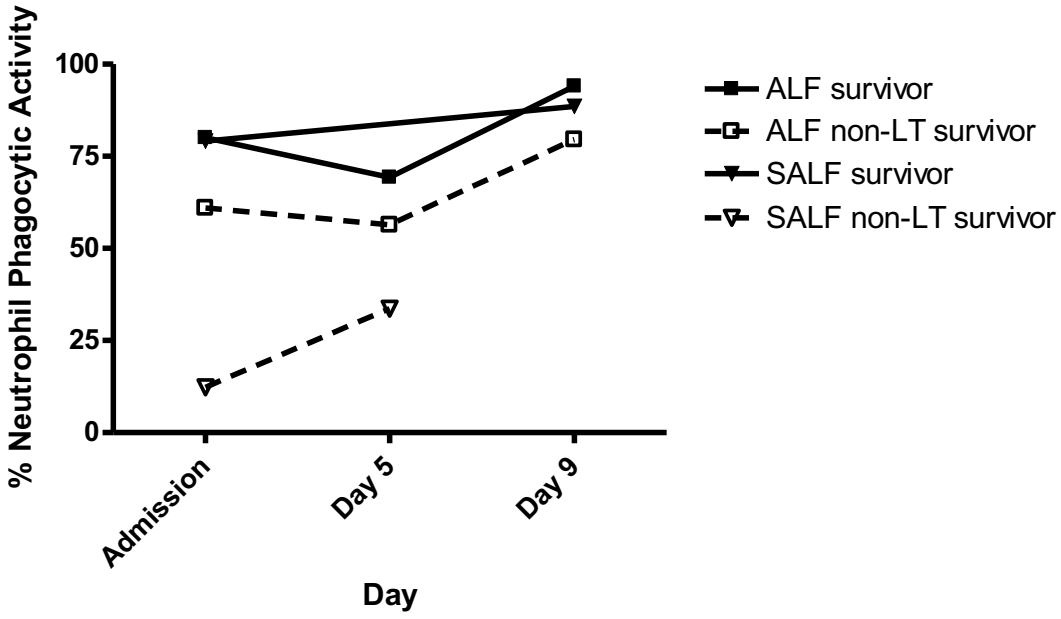


FIGURE 6-4 THE TYPICAL NEUTROPHIL PHAGOCYTIC ACTIVITY TREND OBSERVED IN AN ALF AND SALF SPONTANEOUS SURVIVOR COMPARED TO THAT OBSERVED IN AN ALF (TRANSPLANTED) SURVIVOR AND SALF NON-SURVIVOR ON ADMISSION, DAY 5 AND DAY 9.

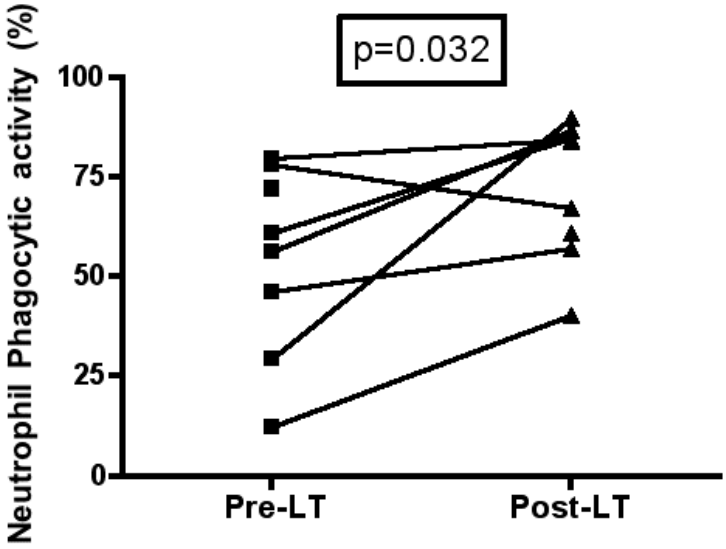


FIGURE 6-5 THE IMPACT OF LIVER TRANSPLANTATION (LT) ON THE MEDIAN NEUTROPHIL PHAGOCYTIC ACTIVITY (%) ON 6 ALF/SALF PATIENTS WHO UNDERWENT LT; P=0.032 USING WILCOXON MATCHED PAIRS TEST

6.6 Neutrophil oxidative burst (OB)

Neutrophil spontaneous production of ROS (SOB) was increased in the patients with ALF compared to HC who went on to require LT, which was reversed within 72 hours post-LT [Table 6-5]. However, SOB was statistically unchanged overall when the ALF/SALF cohorts were compared with the HC and SC groups (p=0.11) [Figure 6-6]. No difference in neutrophil SOB was seen when comparing AALF to non-AALF aetiologies (p=0.99) but an increase in SOB was observed on days 5-9 of ICU admission (p=0.013) with improvement in SOB in those surviving beyond 10 days and 72-hours post-LT [Figure 6-7].

Neutrophil *E. coli* stimulated oxidative burst (ESOB) was significantly reduced in the SC cohort (p<0.05) whilst ALF/SALF neutrophils killed *E. coli* as effectively as HC [Figures 6-8 and Table 6-4].

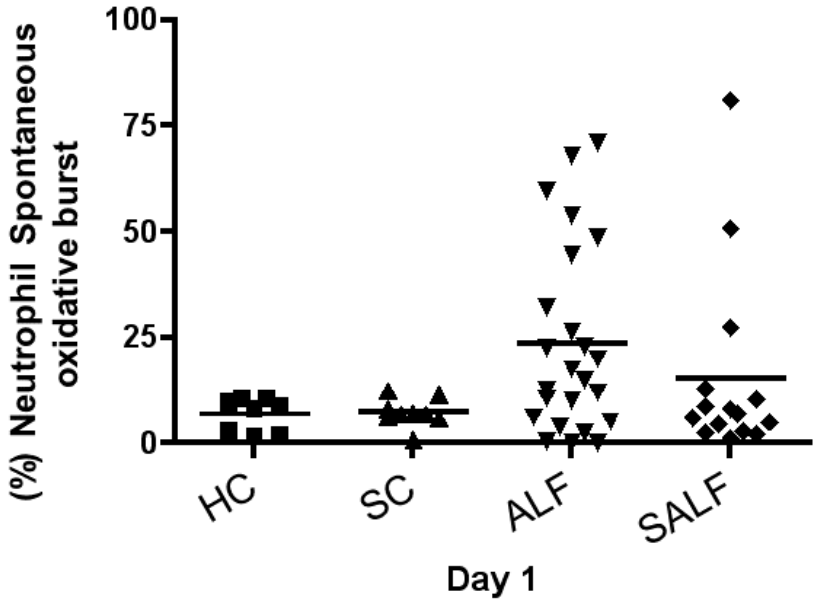


FIGURE 6-6 MEDIAN NEUTROPHIL SPONTANEOUS OXIDATIVE BURST OF HEALTHY CONTROLS (HC), SEPTIC CONTROLS (SC), PATIENTS WITH ACUTE LIVER FAILURE (ALF) AND SUB-ACUTE LIVER FAILURE (SALF).

Differences between groups were calculated using the Kruskal-Wallis test (p=0.11) with Dunn's multiple comparison test (all comparisons p>0.05).

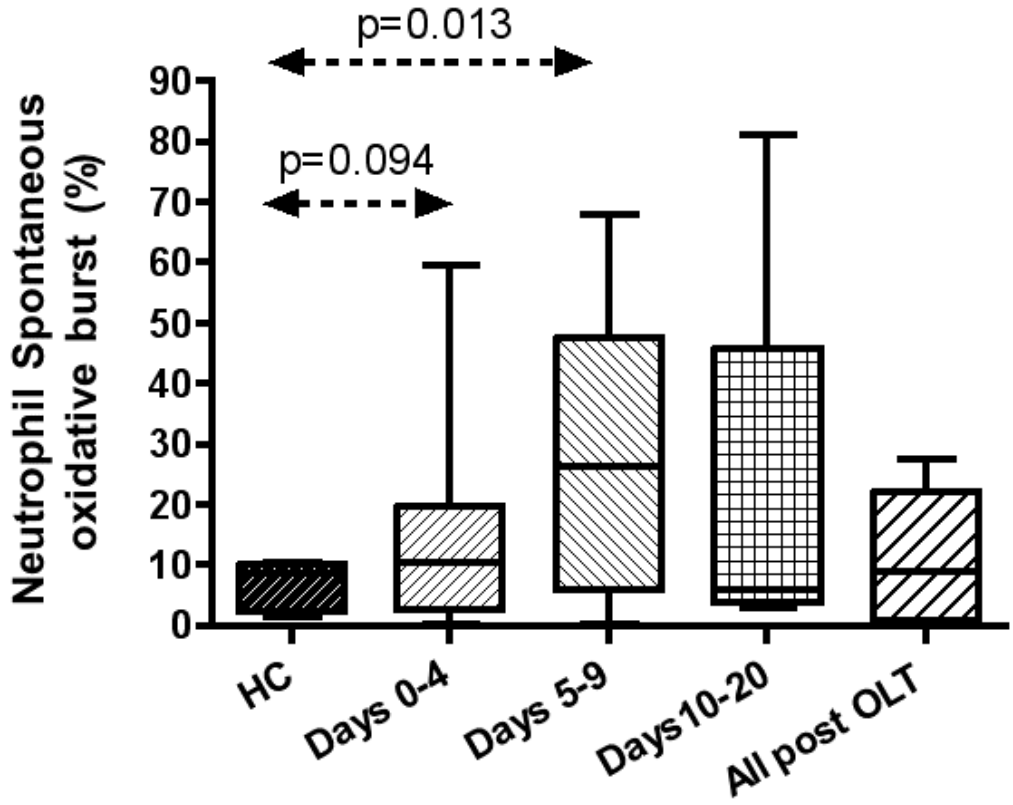


FIGURE 6-7 DETERIORATION IN NEUTROPHIL SPONTANEOUS OXIDATIVE BURST (SOB) IN PATIENTS WITH ACUTE / SUBACUTE LIVER FAILURE COMPARED TO HEALTHY CONTROLS AND PATIENTS WITH ALF/SALF FOLLOWING LIVER TRANSPLANTATION

Differences between groups were calculated using Mann-Whitney U test. Neutrophil SOB was significantly increased compared to healthy controls at days 0-4 (p=0.094) and days 5-9 (P=0.013).

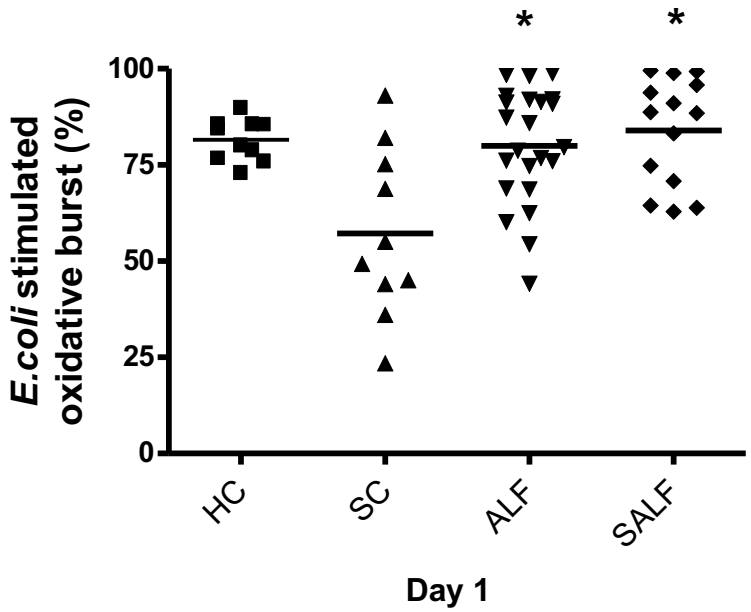


FIGURE 6-8 MEDIAN NEUTROPHIL STIMULATED OXIDATIVE BURST WITH OPSONISED *E COLI* OF HEALTHY CONTROL (HC, N=11) AND SEPTIC CONTROL (SC, N=6) COMPARED TO ACUTE LIVER FAILURE (ALF, N=15) AND SUBACUTE LIVER FAILURE PATIENTS (SALF, N-10)

Differences between groups were calculated using the Kruskal-Wallis test ($p=0.014$) with Dunn's multiple comparison test. The SC group had significantly lower stimulated oxidative burst with opsonised *E coli* compared to both ALF and SALF groups (both $p<0.05$ *).

6.7 Neutrophil function, organ failure scores and plasma-derived factors in ALF and SALF

In the ALF cohort, there was no association seen between neutrophil function and SIRS score, MELD and SOFA score and absolute neutrophil count. Patients with AALF (hyperacute) had higher plasma levels of the pro-inflammatory cytokines TNFα, IL-6 and IL-8 (all $p<0.05$) compared to non-AALF [Figure 6-9]. IL-17 was significantly elevated in the AALF patients who died or underwent LT compared to spontaneous survivors ($p=0.008$) [Table 6-4]. In the ALF cohort spontaneous SOB did not correlate with serum biochemistry, arterial ammonia or organ failure scores.

In the SALF cohort, decreasing NPA correlated with increasing peak arterial ammonia concentration ($p=0.001$; $r^2=0.677$) [Figure 6-10], increasing concentration of plasma IL-10 ($p=0.019$; $r^2=0.407$) [Figure 6-11] and plasma IL-17 ($p=0.0003$; $r^2=0.708$) [Figure 6-12]. In the SALF cohort, increased neutrophil ROS production correlated with higher serum high-density lipoprotein levels ($p=0.001$; $r^2=0.763$) but showed no significant association with organ failure scores.

When ESOB was impaired in the ALF cohort it correlated with lower plasma concentrations of IL-6 ($p=0.038$; $r^2=0.190$), IL-10 ($p=0.047$; $r^2=0.175$) and IL-17 ($p=0.007$; $r^2=0.301$) [data not shown] and in the SALF cohort, correlated with higher plasma concentrations of IL-8 ($p=0.007$; $r^2=0.465$) [Figure 6-13] and lower plasma concentration of IL-17 ($p=0.025$; $r^2=0.354$) [data not shown].

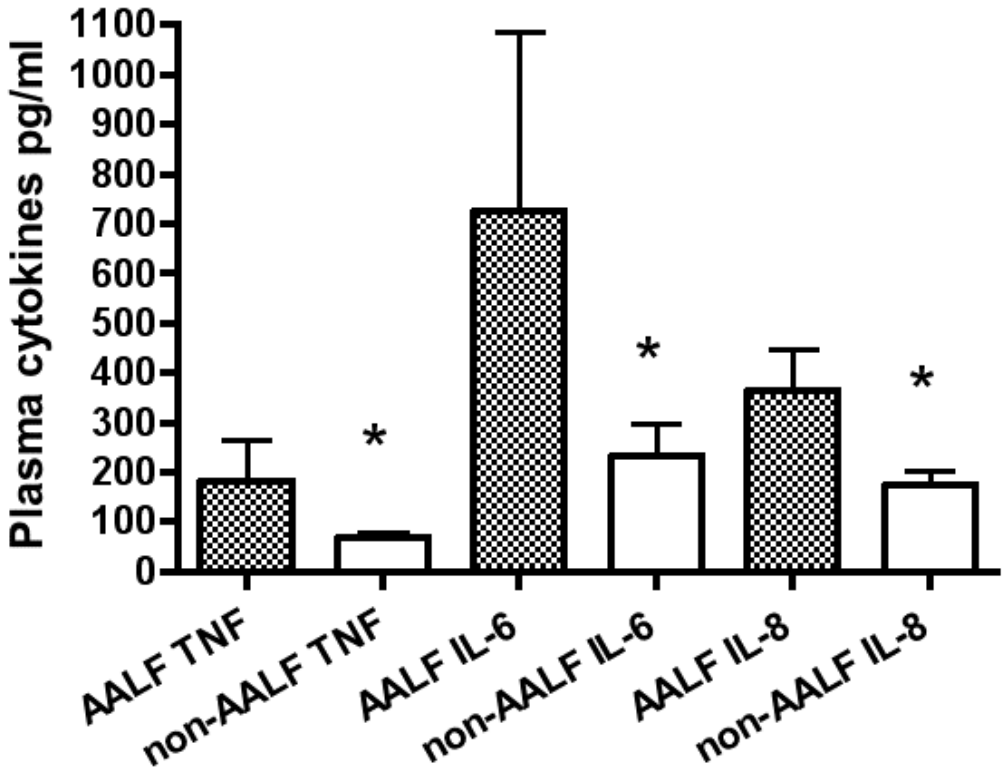


FIGURE 6-9 COMPARISON OF INFLAMMATORY CYTOKINE LEVELS IN NEUTROPHILS FROM PATIENTS WITH ACETAMINOPHEN-INDUCED (AALF) AND NON-ACETAMINOPHEN INDUCED (NON-AALF) ACUTE LIVER FAILURE

Difference between groups were calculated using Mann-Whitney U test, *$p<0.05$.

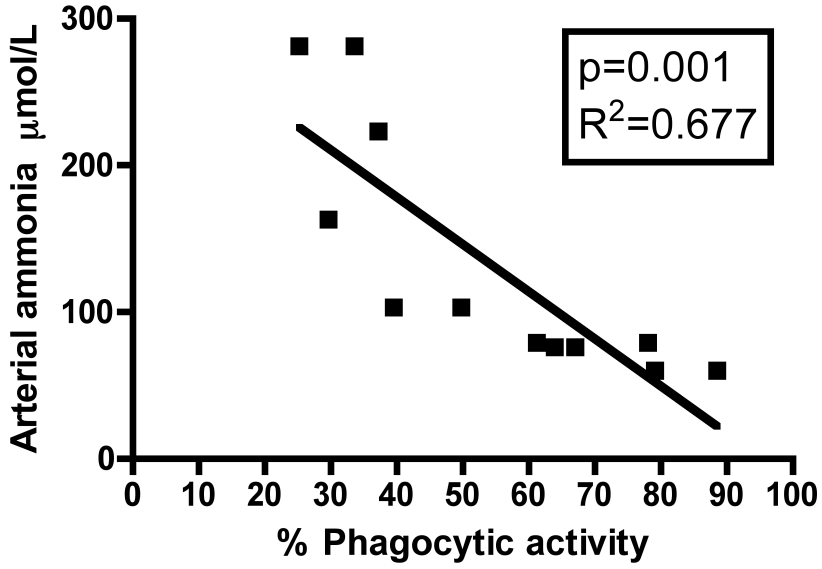


FIGURE 6-10 CORRELATION BETWEEN IMPAIRED NEUTROPHIL PHAGOCYTIC ACTIVITY (%) AND ARTERIAL AMMONIA (µMOL/L) IN THE SUBACUTE LIVER FAILURE COHORT (R^2=0.677; P=0.001).

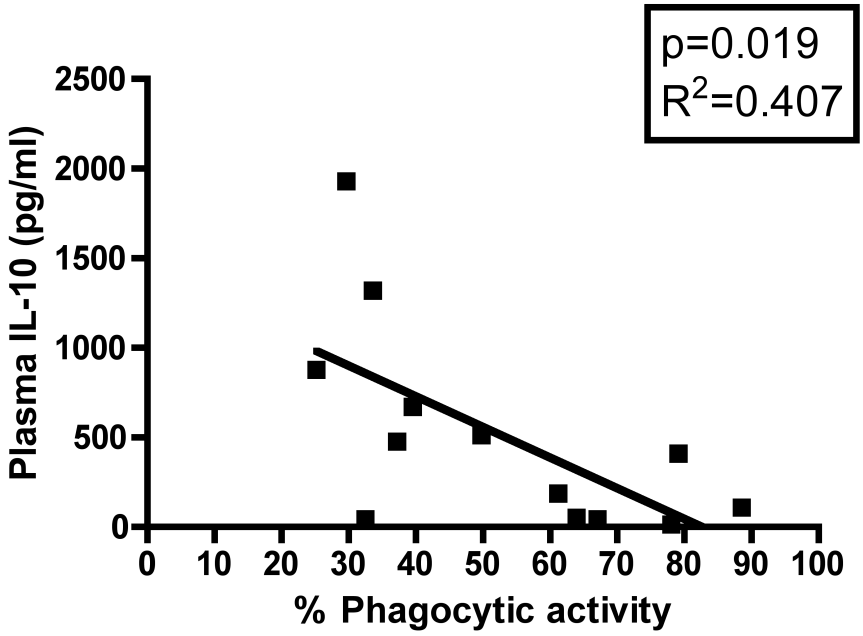


FIGURE 6-11 CORRELATION BETWEEN PERCENTAGE NEUTROPHIL PHAGOCYTIC ACTIVITY AND PLASMA IL-10 CONCENTRATION (PG/ML) IN PATIENS WITH SUBACUTE LIVER FAILURE

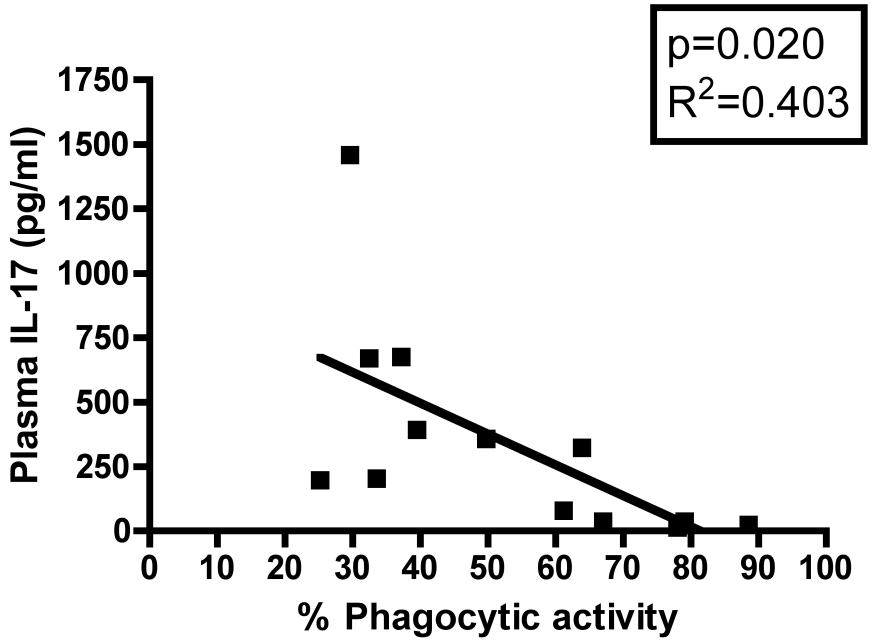


FIGURE 6-12 THE CORRELATION BETWEEN IMPAIRED NEUTROPHIL PHAGOCYTIC ACTIVITY (%) AND PLASMA IL-17 (PG/ML) IN SUBACUTE LIVER FAILURE

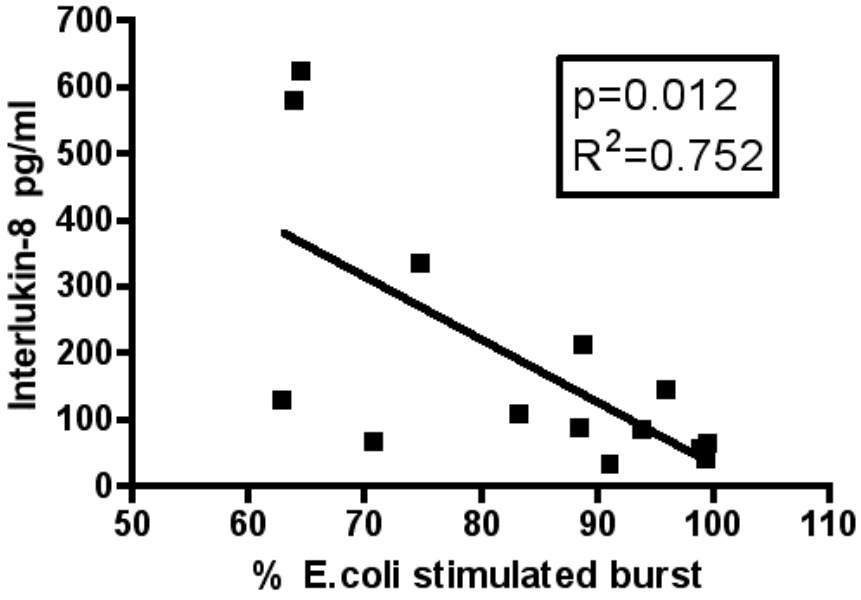


FIGURE 6-13 THE CORRELATION BETWEEN IMPAIRED NEUTROPHIL E.COLI STIMULATED OXIDATIVE BURST AND IL-8 PG/ML IN THE SALF COHORT

6.8 Neutrophil Function and Patient Outcomes

Patients who died and those proceeding to LT had lower NPA on day 1 than spontaneous survivors (p=0.047) [Table 6-7 and Figure 6-14]; however, this was not maintained after exclusion of patients undergoing LT (p=0.19). No difference was observed when comparing admission neutrophil spontaneous OB in spontaneous survivors to patients who died or underwent LT (p=0.5) [Table 6-7].

Four deaths occurred in the ALF cohort; 3 patients died of MODS (two on day 10 of ICU admission and one on day 45) and 1 died of uncontrolled intracranial hypertension. In the SALF cohort, 2 patients died from MODS on days 15 and 17 of the ICU admission, respectively. The incidence of culture positive sepsis in the ALF/SALF cohorts overall was low with one patient with seronegative SALF developing an episode of *staphylococcus epidermidis* bacteraemia on day 3 (day 1 NPA 29.7% improving to 40.3% post LT on day 6) and in another seronegative SALF a *Klebsiella species* urinary tract infection developed on day 18 (patient had a NPA of 61%).

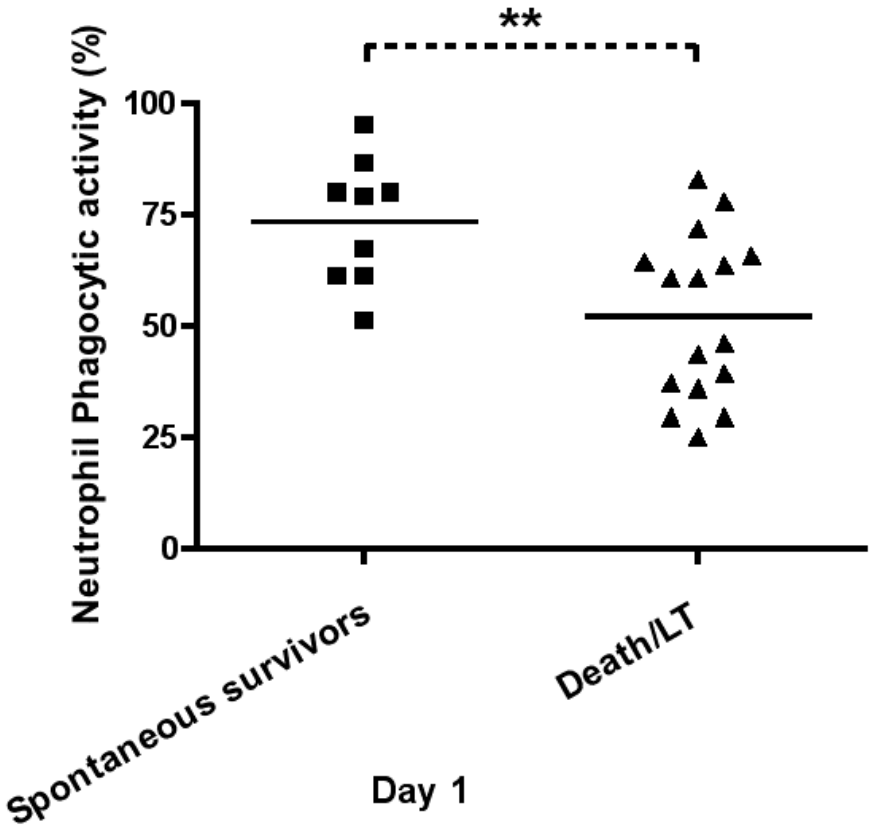


FIGURE 6-14 MEDIAN PERCENTAGE NEUTROPHIL PHAGOCYTIC ACTIVITY OF PATIENTS WITH ACUTE/SUBACUTE LIVER FALURE WHO SPONTANEOUSLY SURVIVED (9 PATIENTS) WITHOUT LIVER TRANSPLANTATION (LT) COMPARED TO THOSE WHO DIED OR UNDERWENT LT (10 PATIENTS)

p=0.008 ** using Mann Whitney test.

6.9 Discussion

Neutrophil dysfunction has been implicated both in the immune paresis observed in ALF (Altin et al., 1983, Clapperton et al., 1997) and in direct injury to the liver (Ramaiah and Jaeschke, 2007) and extra-hepatic organs. (Awad et al., 2009) Neutrophil driven hepatocellular injury has also been shown to contribute to hepatocellular damage in models of ischemia-reperfusion injury, (Jaeschke, 2006) alcoholic hepatitis (Bautista, 2002) and endotoxemia. (Dorman et al., 2005) Neutrophil recruitment is seen within the liver in AALF (Lawson et al., 2000) and there is evidence to suggest that hepatocyte injury is amplified by neutrophil infiltration in a mouse model of AALF. (Liu et al., 2004b) Neutrophils are also likely to contribute to liver injury in ALF, resulting from their overwhelming capacity to produce large quantities of ROS and proteases following recruitment and activation within the hepatic sinusoids. For this reason, the relationship between circulating neutrophil function and the progression and outcomes of acute liver injury was therefore explored in this study.

The observation of a reduction in NPA and elevated SOB in ALF/SALF cohorts, akin to that frequently observed in sepsis, (Brown et al., 2006) may explain why patients with ALF exhibit phenotypic features of septic shock with microvascular dysfunction, hemodynamic instability, coagulopathy, encephalopathy, MODS and high levels of circulating pro-inflammatory cytokines. Why the severity of NPA is less so in those presenting with ALF compared to SALF is less clear but the development of impaired NPA may occur in a time dependent manner as evidenced by the most severe reduction in phagocytic ability seen in cases of SALF, where the liver injury takes on a more insidious course over several weeks. Indeed, many patients with established SALF may present with moderate portal hypertension with features of splenomegaly and ascites. Nevertheless, NPA on admission appears to be a predictor of spontaneous survival compared to conventional organ failure scores such as SOFA and MELD, which did not predict poor outcome in this study. Trying to understand the relationship between neutrophil phagocytic dysfunction and poor prognosis therefore seems critical. LT resulted in rapid improvement of neutrophil phagocytic function within 72 hours but not complete reversal, which could be the result of ischemia-reperfusion phenomena, ongoing production of pro-inflammatory cytokines/SIRS or sepsis.

The incidence of ‘culture positive’ sepsis was low in this ALF/SALF cohort overall and indeed the observed deaths could not be directly attributed to infection,

suggesting phagocytic dysfunction is either a reflection of general immune activation or a specific factor related to liver failure. Peak plasma ammonia levels demonstrated a robust correlation with poor phagocytic function in SALF and high circulating levels of IL-10 and IL-17. Ammonia was previously shown to impair NPA and induce SOB in healthy neutrophils exposed to supraphysiological concentrations of ammonia *ex vivo* and in rats fed an ammonia-rich diet. (Shawcross et al., 2008a) The peak arterial ammonia concentration did not however correlate with impaired NPA in the ALF cohort but might be attributed to the impact of ammonia reduction by continuous veno-venous hemofiltration prior to neutrophil sampling.

Pro- and anti-inflammatory cytokine profiles might be expected to show a closer association with neutrophil OB than phagocytic activity although this was not generally the case. Higher plasma IL-10 and IL-17 concentrations correlated with impaired NPA and may suggest the development of a CARS in this condition (Antoniades et al., 2008) with involvement of T-regulatory cells. (Lewis et al., 2012) However, the condition with the highest levels of pro-inflammatory cytokines, AALF demonstrated only modest neutrophil dysfunction. CD4+CD25+CD127-FOXP3+ T-regulatory cells directly inhibit neutrophil function, promoting apoptosis and death when exposed to lipopolysaccharide through TLR4 expressed on their surface which inhibits pro-inflammatory activities. (Lewkowicz et al., 2006) This is an important role in the direct control of innate immune responses. Upon activation, these T-regulatory cells can either induce themselves or CD4+CD25-FOXP3-T effector cells to differentiate into IL-17A producing cells (Th17) in the presence of TGF-beta and/or IL-6. (Xu et al., 2007) In contrast to the role of T-regs on neutrophils, one of the functions of Th17 is to recruit neutrophils into inflamed tissue further increasing the antimicrobial response *in vitro* and *in vivo*. (Annunziato et al., 2008, Kolls et al., 2008) The balance between Th-17 and T-regulatory cells are important in modulating innate immune response in the liver and they have been shown to play an important role in acute and chronic hepatitis B virus infection as well as autoimmune hepatitis although little is know about their role in ALF. (Xue-Song et al., 2012, Grant et al., 2014)

The evidence for a role of increased circulating neutrophil production of ROS as a contributor to the development of MODS and poor outcomes in ALF in this study is less clear than that of NPA. Interestingly, in the SALF cohort increased SOB correlated with increased serum high-density lipoprotein levels and higher SOFA and APACHE II scores. High-density lipoprotein plays an important role in the transport of cholesterol to

the adrenal gland for steroidogenesis thus modulating the response to sepsis and critical illness. Low concentrations of high-density lipoprotein have recently been shown to be a predictor of poor outcome in ALF but were not associated with an increased risk of sepsis. (Etogo-Asse et al., 2012) The problem with measuring spontaneous neutrophil ROS production in isolated circulating cells is that this may not reflect the production within the hepatic parenchyma or other organs so it is difficult to draw firm conclusions. In addition, ALF and SALF patients are a heterogeneous patient group who are prone to deteriorating rapidly necessitating a number of invasive interventions such as high flow hemofiltration and mild hypothermia potentially influencing neutrophil function and which are difficult to control for constituting the main weakness of this study. Furthermore, the empirical use of potent broad-spectrum antibiotics and anti-fungal agents as standard of care in this study is also likely to have abrogated any increased susceptibility to developing sepsis in this cohort.

Neutrophil stimulated OB with *E. coli* was significantly reduced in the SC group, whilst ALF/SALF neutrophils killed *E. coli* as effectively as HC. This may represent the fact that neutrophils in patients with sepsis have been exhausted fighting the infection and have very little capacity left for responding to the *E. coli*. Alternatively, it could result from the development of CARS.

In summary, circulating neutrophils in patients with ALF/SALF have impaired bacteriocidal function similar to that seen in patients with severe sepsis and MODS. Neutrophil function indices are important biomarkers of poor prognosis in ALF/SALF and can be implicated as important mediators in the development of cellular and organ dysfunction and the increased susceptibility to developing sepsis. Clearly these neutrophil function tests in their present format are cumbersome to perform and cannot be performed at the bedside but development of a rapid test of neutrophil dysfunction may offer the possibility for refinement of current prognostic criteria and might tailor therapy to those at highest risk. These data also support the circulating neutrophil as a novel therapeutic target in ALF.

Chapter 7 –The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality.

7.1 Introduction

Cirrhosis is associated with an increased incidence of microbial infection resulting in hospitalisation and complicating hospital admissions in up to 40% of cases. (Fernandez et al., 2002) Infection can lead to worsening liver function and precipitate complications, including variceal bleeding, HE, AKI and MODS, contributing to high mortality. The exponential increase in the incidence of cirrhosis in the United Kingdom (BASL and BSG, 2009) poses a major challenge in managing these patients particularly with respect to utilisation of ICU beds and requirement for LT. With poor outcomes following sepsis and its associated complications, and increasing waiting list mortality for LT, there is an urgent need for novel approaches to reducing the rate of infection in cirrhosis.

Cirrhosis represents a state of functional immune paresis with defects in opsonisation, innate and adaptive immune responses and up-regulation of pro-inflammatory cytokine production akin to that observed in patients with sepsis. (Wasmuth et al., 2005) Neutrophils are key effector cells of the innate immune response and show defects in phagocytic capacity and OB in cirrhosis. (Shawcross et al., 2008a, Rajkovic and Williams, 1986) In patients with cirrhosis bacterial translocation, porto-systemic shunting and reduced reticulo-endothelial function culminate in increased levels of circulating endotoxin and other PAMP molecules. PAMPs interact with PRR, such as Toll-like receptors (TLR), expressed on the neutrophil surface leading to in the priming of circulating neutrophils. (Sabroe et al., 2003) Neutrophil priming also occurs in response to circulating chemokines and pro-inflammatory cytokines. (Condliffe et al., 1998) In the primed state circulating neutrophils show altered surface receptor expression, increased basal superoxide production and reduced thresholds for undergoing phagocytosis and OB. (Colotta et al., 1992, Hayashi et al., 2003) Primed circulating neutrophils have been postulated to play a central role in the development of acute kidney and lung injury in sepsis, (Brown et al., 2006, Awad et al., 2009) as well as accumulating in other organs in patients who died of MODS. (Nuytinck et al., 1987) The generation and release of ROS and granule contents, such as serine proteases (e.g. elastase and MMP-9) by neutrophils at these sites result in tissue damage and the development of MODS. (Smith, 1994)

Antibiotic prophylaxis is inadequate at reducing the incidence of infection in patients with cirrhosis and may be associated with the development of multi-drug resistant organisms. (Fernandez et al., 2002) A greater understanding of the pathogenic

mechanisms underpinning neutrophil dysfunction in cirrhosis might facilitate better identification of those at risk of developing infection and promote the development of neutrophil-targeted therapies.

The aim of this longitudinal prospective study was to investigate whether defective neutrophil functioning in patients with cirrhosis predicts the development of infection and extra-hepatic organ dysfunction. To test the hypothesis we characterised neutrophil phenotype and functional capacity in a large cohort of cirrhotic patients in relation to clinical characteristics, plasma biochemical and cytokine profile and the development of infection and organ dysfunction at 90-days and 1-year.

7.2 Patients recruited

From October 2008 to August 2010 a total of 67 patients were recruited to the following cohorts: 49 patients with stable cirrhosis (CLD); 12 patients with acute-on-chronic liver failure (AoCLF) as previously defined by Sen et al (Sen et al., 2002); and 13 patients with hepatic encephalopathy (HE) and are detailed in Table 7-1. Three patients with stable cirrhosis subsequently developed either AoCLF (n=2) or HE (n=1). Results of the investigations from these cohorts were compared with 11 healthy controls (HC) and 6 septic controls (SC) recruited concomitantly during this time period.

Baseline demographic, biochemical and physiological parameters are detailed in Tables 7-1 and 7-2. The aetiology of cirrhosis across the 3 groups was: alcohol-related liver disease (ARLD) 44.8% (30/67); chronic viral hepatitis 16.4% (11/67); autoimmune liver disease (including autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis) 22.4% (15/67); other 16.4% (11/67). Aetiology of liver disease in the other group comprised cryptogenic cirrhosis (n=4), non-alcoholic steatohepatitis (n=3), haemochromatosis (n=2), and Budd-Chiari syndrome (n=2). In the alcohol group 14/26 (54%) patients were consuming alcohol prior to hospital admission. In patients with alcohol-related cirrhosis who were abstinent from alcohol 12/26 (46%), the median (range) length of abstinence was 12 months (5-120 months).

A number of significant differences were observed in baseline biochemical variables between the individual cohorts. The haemoglobin level and platelet count were lower in the AoCLF cohort compared to stable cirrhosis ($p<0.01$ and $p<0.05$, respectively) whereas neutrophil count ($p<0.01$), CRP ($p<0.05$), serum creatinine ($p<0.001$), bilirubin ($p<0.001$), INR ($p<0.001$), aspartate aminotransferase (AST)

($p<0.01$), MELD, SOFA and APACHE II (all $p<0.001$) were all significantly higher in the AoCLF cohort [Table 7-2].

The median (range) length of follow-up, including deaths, in the stable cirrhosis and AoCLF cohorts was 282 days (1-1021) and 12 days (0-805), respectively. Five patients were lost to follow-up in the stable cirrhosis group at a median of 48 days (1-282). During the study follow-up 7 deaths occurred in the stable cirrhosis cohort, 2 from sepsis, at a median of 77 days. 16 patients with stable cirrhosis underwent LT at a median of 34 days and 26 patients survived to 12 months without LT [Figure 7-1]. In the AoCLF cohort 9 patients died, 6 from MODS secondary to bacterial and fungal infection, at a median of 12 days. Three AoCLF patients underwent LT all within 45 days and one patient survived an episode of peritonitis to discharge from hospital [Figure 7-1].

Table 7-1 Characteristics of patients with cirrhosis.

	Healthy Controls (n=11)	**Cirrhosis † (n=49)**	**HE (n=13)**	**AoCLF (n=12)**
Median Age (range)	33 (30.5-33.5)	52 (43-61)	57 (45-61)	44 (39-62)
Female	3	13 (27)	4 (31)	4 (33)
Aetiology				
Alcohol		19 (39)	9 (69)	5 (42)
Viral hepatitis		10 (20)	0	1 (8)
Autoimmune liver disease		10 (20)	2 (15)	5 (42)
Other		10 (20)	2 (15	1 (8)
Varices		7 (14)	7 (54)	6 (50)
Ascites		34 (69)	10 (77)	11 (92)
Diuretic resistant ascites		9 (18)	5 (38)	7 (58) §
Hepatic encephalopathy		0	13 (100)	9 (75)
Grade 1-2		0	10	2
Grade 3-4		0	3	7
Antibiotics		16 (33)	10 (77) §§	11 (92) §§§
Active infection		0 (0)	3 (23) §	7 (58) §§
B-blockers		23 (47)	3 (23)	2 (17)
Ventilated		0	0	6 (50)
Vasopressors		0	0	6 (50)
CVVH		0	0	8 (75)
1 year LT free survival		28 (57)	6 (46) §	1 (8) §§§
90 day LT free survival		34 (69)	6 (45) §	1 (8) §§§
Transplanted within 1-year		14 (29)	2 (15)	3 (25)

Data presented as number of cases (%) or median (IQR) for the variables as appropriate. p-values calculated using Chi-squared for 2 groups and Kruskal-Wallis for 3 or more groups with Dunn's multiple comparison test; p value <0.05 considered significant, § $p \leq 0.05$ versus cirrhosis, §§ $p \leq 0.01$ versus cirrhosis, §§§ $p \leq 0.001$ versus cirrhosis.

Abbreviations: AoCLF: acute-on -chronic liver failure; CVVH: continuous veno-venous haemofiltration; HE: hepatic encephalopathy; IQR: interquartile range; LT: liver transplant. † Stable cirrhosis with no evidence of overt hepatic encephalopathy or organ dysfunction/failure.

Table 7-2 Clinical, biochemical, and organ failure parameters of patients with cirrhosis.

	Cirrhosis N=49	**HE N=13**	**AoCLF N=12**	**p-values**
Haemoglobin (g/L)	105 (95-127)	84 (80-93) §	84 (55-103) §§	<0.001
Neutrophil count (x 10^9/L)	3.2 (2.0-4.4)	4.0 (2.9-8.8)	9.7 (7.6-15.6) §§	<0.001
Platelets (x 10^9/L)	103 (81-155)	111 (68-128)	77 (58-104)	0.193
C-reactive protein (mg/L)	9 (5-25)	22 (13-44)	52 (29-93) §§	0.003
Sodium (mmol/L)	137 (134-139)	135 (133-135)	137 (134-142)	0.269
Creatinine (μmol/L)	80 (70-88)	93 (84-122)	180 (150-210) §§	<0.001
Bilirubin (μmol/L)	38 (24-84)	134 (73-350)	432 (293-553) §§	<0.001
Albumin (g/L)	32 (28-38)	28 (24-35)	27 (26-34)	0.066
INR	1.42 (1.24-1.71)	1.9 (1.5-2.3) §	2.4 (1.9-2.9) §§	<0.001
Aspartate Aminotransferase (IU/L)	58 (45-102)	77 (50-94)	116 (81-186) §	0.020
Arterial ammonia (μmol/L)	75.5 (52-122)	71 (58-92)	69 (55-103)	0.786
Child-Pugh score (IQR)	8 (7-11)	12 (10-13) §§	12.5 (11-13) §§	<0.001
Child-Pugh class A (%)	12 (24)	1 (8)	0	
Child-Pugh class B (%)	19 (39)	2 (15)	0	
Child-Pugh class C (%)	18 (37)	10 (77)	12 (100)	
MELD	14 (11-20)	24 (19-33) §	34 (30-38) §§	<0.001
UKELD	54 (51-58)	60 (57-64) §	63 (60-68) §§	<0.001
APACHE II	9 (7-11)	10 (6-11)**	21.5 (19-25) §§	<0.001
SOFA	3 (2-5)	5 (4-7)**	15 (11-17) §§	<0.001

Data presented as number of cases (percentage) or median (IQR) for the variables as appropriate. p-values calculated using Kruskal-Wallis with Dunn's multiple comparison test; § $p \leq 0.05$ versus cirrhosis, §§ $p \leq 0.01$ versus cirrhosis, ** $p \leq 0.01$ versus AoCLF.

Abbreviations: AoCLF: acute-on-chronic liver failure; HE: hepatic encephalopathy; IQR: interquartile range; MELD-Model of End-stage liver disease; SOFA-Sequential Organ Failure Score; APACHE II-Acute Physiology and Chronic Health Evaluation II. UKELD: United Kingdom Model for End-Stage Liver Disease.

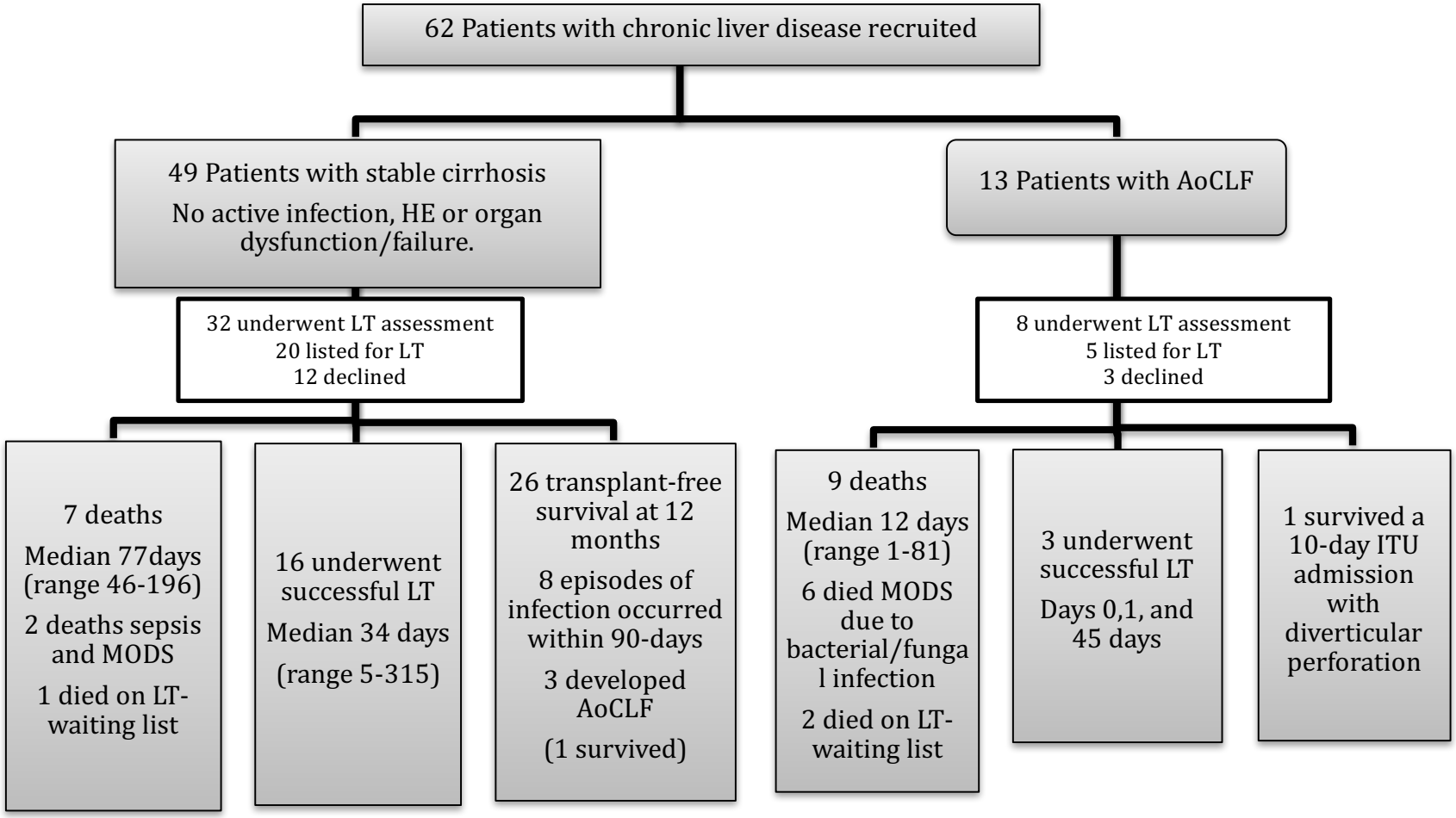


FIGURE 7-1 FLOW DIAGRAM TO ILLUSTRATE THE OUTCOMES OF THE 62 PATIENTS WITH CIRRHOSIS RECRUITED TO THE STUDY.

Abbreviations: AoCLF: acute-on-chronic liver failure; LT: liver transplantation; MODS: multiple organ dysfunction syndrome

7.3 Study to characterise neutrophil phenotype, function and morphology in chronic liver disease

7.3.1 Neutrophil phenotype

Baseline neutrophil surface receptor expression of CD16 (FcγRIII) was unchanged in patients with cirrhosis compared to HC (p=0.544) [Figure 7-2]. Neutrophil surface receptor expression of CD11b (Mac-1) in patients with cirrhosis increased compared to HC (p=0.023) [Figure 7-2]. Reduced neutrophil CD62L expression was found in 2 out of 3 patients with cirrhosis in the chemotaxis study [Table 7-5] indicating an activated phenotype.

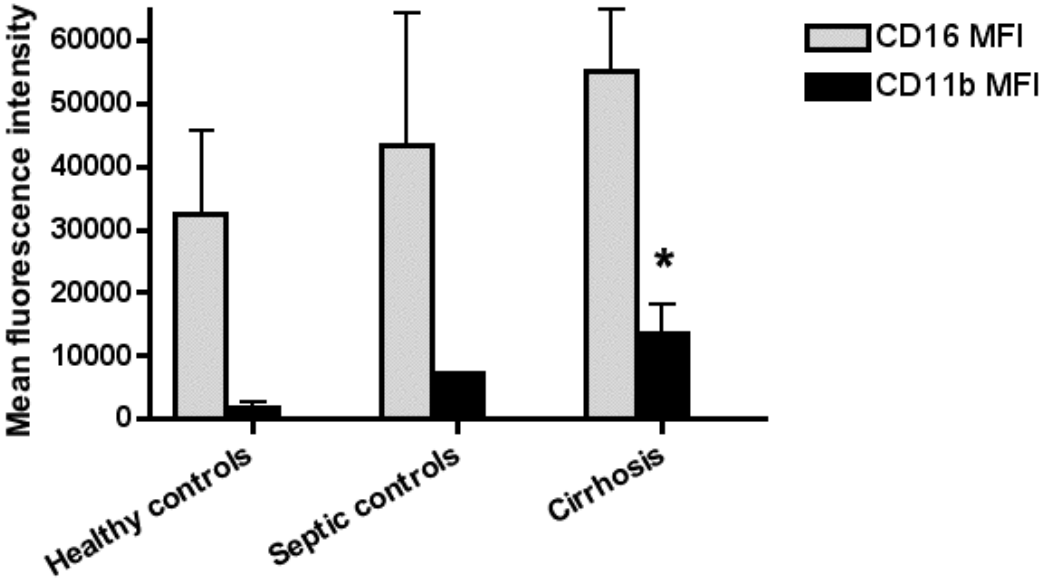


FIGURE 7-2 NEUTROPHIL SURFACE CD16 (IMMUNOGLOBULIN G: FCγRIII) AND CD11B EXPRESSION MEASURED BY MEAN FLUORESCENCE INTENSITY (MFI) IN HEALTHY CONTROLS, SEPTIC CONTROLS AND PATIENTS WITH CIRRHOSIS.

Differences between healthy controls and patients were calculated using the Kruskal-Wallis test with Dunn's test for multiple comparisons. Patients with cirrhosis but not septic controls show increased CD11b expression compared to HC (p=0.023).

7.3.2 Neutrophil phagocytic activity (NPA)

Overall NPA was significantly impaired in patients with cirrhosis compared to HCs (p=0.002) [Table 7-3 and Figures 7-3 and 7-5]. SC showed a tendency to reduced NPA compared to HC (p=0.119) [Table 7-3 and Figures 7-4 and 7-5]. Although there was a tendency for NPA to be reduced in patients with Child-Pugh class A (CPA) cirrhosis, a significant reduction was only observed in patients with Child-Pugh class B (CPB) (p=0.026) and Child-Pugh class C (CPC) cirrhosis (p=0.023) [Figure 7-6].

The CPC cohort was further sub-divided based upon the presence or absence of diuretic refractory ascites (DRA) [DRA being defined as ascites that cannot be mobilized or the early recurrence of which cannot be satisfactorily prevented by medical therapy]. (Arroyo and Colmenero, 2003) DRA patients represent an extreme of physiological adaptation to splanchnic arterial vasodilatation and portal hypertension and two groups are recognised: diuretic resistant ascites where maximal doses of diuretics are ineffective (Spironolactone 400mg once daily and furosemide 160mg per day); diuretic intolerant ascites whereby patients are unable to take an adequate diuretic dose due to development of complications such as AKI or hyponatraemia. Patients with CPC cirrhosis and DRA on non-specific β-blockers had significantly impaired NPA compared to patients not taking the drug ($p<0.05$) [Figure 7-7].

NPA was not influenced by severity of liver disease as determined by the MELD score (p=0.9) or UKELD score (p=0.8). NPA was not influenced by cirrhosis aetiology (p=0.36) [data not shown]. NPA was significantly increased in active drinkers with cirrhosis compared to patients who were abstinent (p=0.029) [Table 7-4 and Figure 7-8].

Table 7-3 Neutrophil function indices and cytokines in healthy controls, septic controls and patients with cirrhosis

Neutrophil function	**Healthy controls N=11**	**Severe sepsis N=6**	**† Cirrhosis N=49**	**p-values**
Neutrophil phagocytic activity (%)	77.1 (72.2-83.6)	68.9 (59.5-78.5)	63.6 (45.5-75.5)**	0.004
Neutrophil phagocytic capacity (*E.coli* MFI)	7.99×10^4 (3.74×10^4-9.08×10^4)	3.45×10^4 (1.99×10^4-4.37×10^4)	3.03×10^4 (1.82×10^4-4.36×10^4)	0.019
Neutrophil spontaneous oxidative burst (%)	8.7 (5.6-9.3)	7.0 (6.4-10.7)	9.1 (5.6-26.5)	0.284
Neutrophil resting forward scatter geometric MFI	7.67×10^4 (7.46×10^4-7.74×10^4)	7.08×10^4 (6.19×10^4-7.29×10^4)	7.13×10^4 (6.77×10^4-7.55×10^4)	
Neutrophil *E.coli* stimulated oxidative burst (%)	84.9 (79.7-85.6)	52.2 (44.3-73.6)*	85.1 (69.3-92.9)	0.011
Neutrophil *E.coli* stimulated rhodamine MFI	1.56×10^4 (1.19×10^4-2.58×10^4)	3.89×10^3 (3.21×10^3-5.12×10^3)	2.72×10^4 (1.62×10^4-4.22×10^4)	0.027
Tumour Necrosis Factor-α pg/mL	9 (3-39)	20 (17-67)	60 (14-384)	0.198
Interlukin-1β pg/mL	4545 (1429-7346)	872 (69-2652)	8416 (2007-29949)	0.003
Interleukin -6 pg/mL	21 (16-38)	96 (75-172)	75 (14-435)	0.781
Interleukin -8 pg/mL	23 (13-34)	65 (34-74)	93 (42-273)	0.380
Interleukin -10 pg/mL	72 (28-76)	78 (20-289)	145 (57-285)	0.022
Interleukin -17 pg/mL	66 (46-163)	78 (20-289)	263 (82-948)	0.017

Data presented as median percentage or mean fluorescence intensity (MFI) and inter-quartile range (IQR) for the variables as appropriate. p-values calculated using the Kruskal-Wallis test with Dunn's test for multiple comparisons or Mann-Whitney U test as appropriate. *$p<0.05$, ** $p < 0.01$ versus healthy controls. † stable cirrhosis with no evidence of overt hepatic encephalopathy or organ dysfunction/failure.

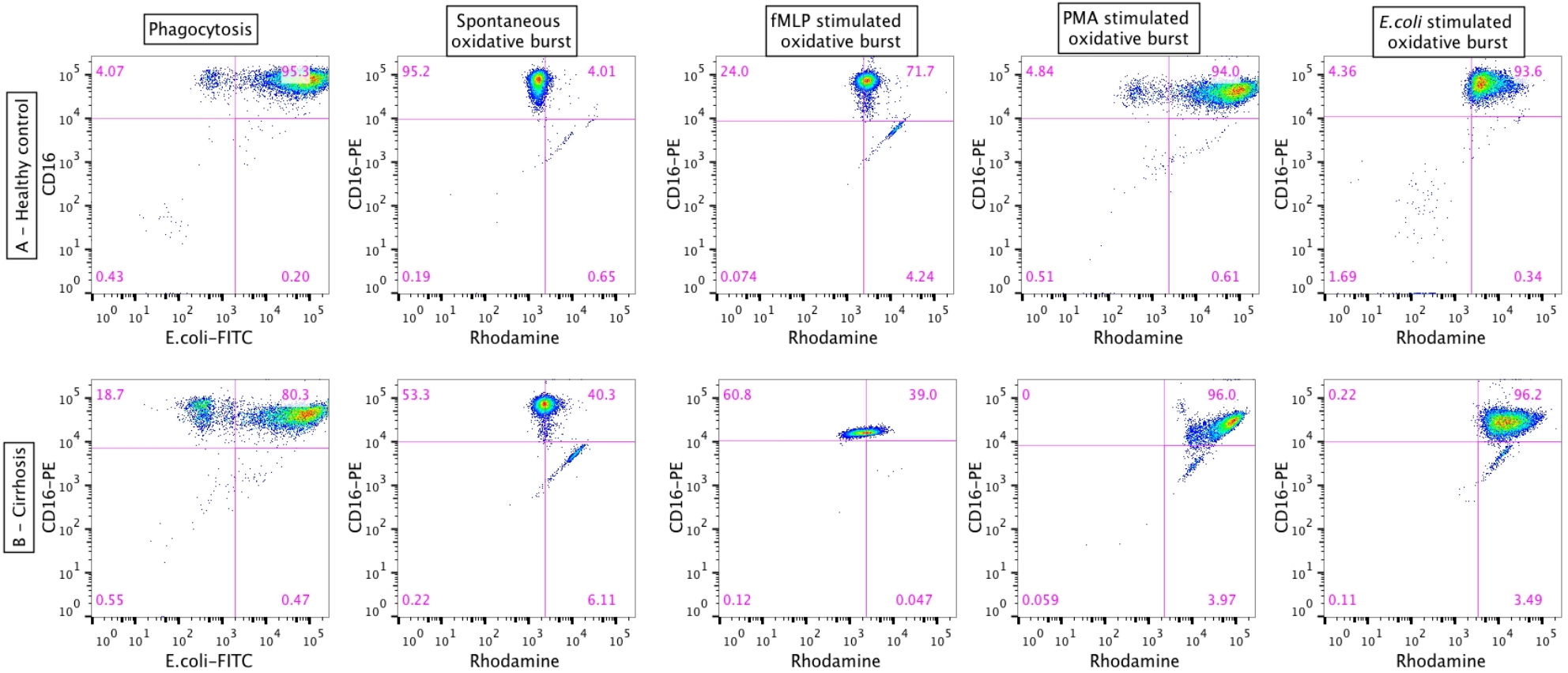


FIGURE 7-3 FLOW CYTOMETER PLOTS OF NEUTROPHIL FUNCTION IN A HEALTHY CONTROL (HC) AND A PATIENT WITH STABLE CIRRHOSIS.

Abbreviations: fMLP: Formyl-methionyl-leucyl-phenylalanine; PMA: Phorbol myristate acetate; CD16 PE: Cluster of differentiation 16 – Phycoerytherin; FITC: Fluorescein isothiocyanate.

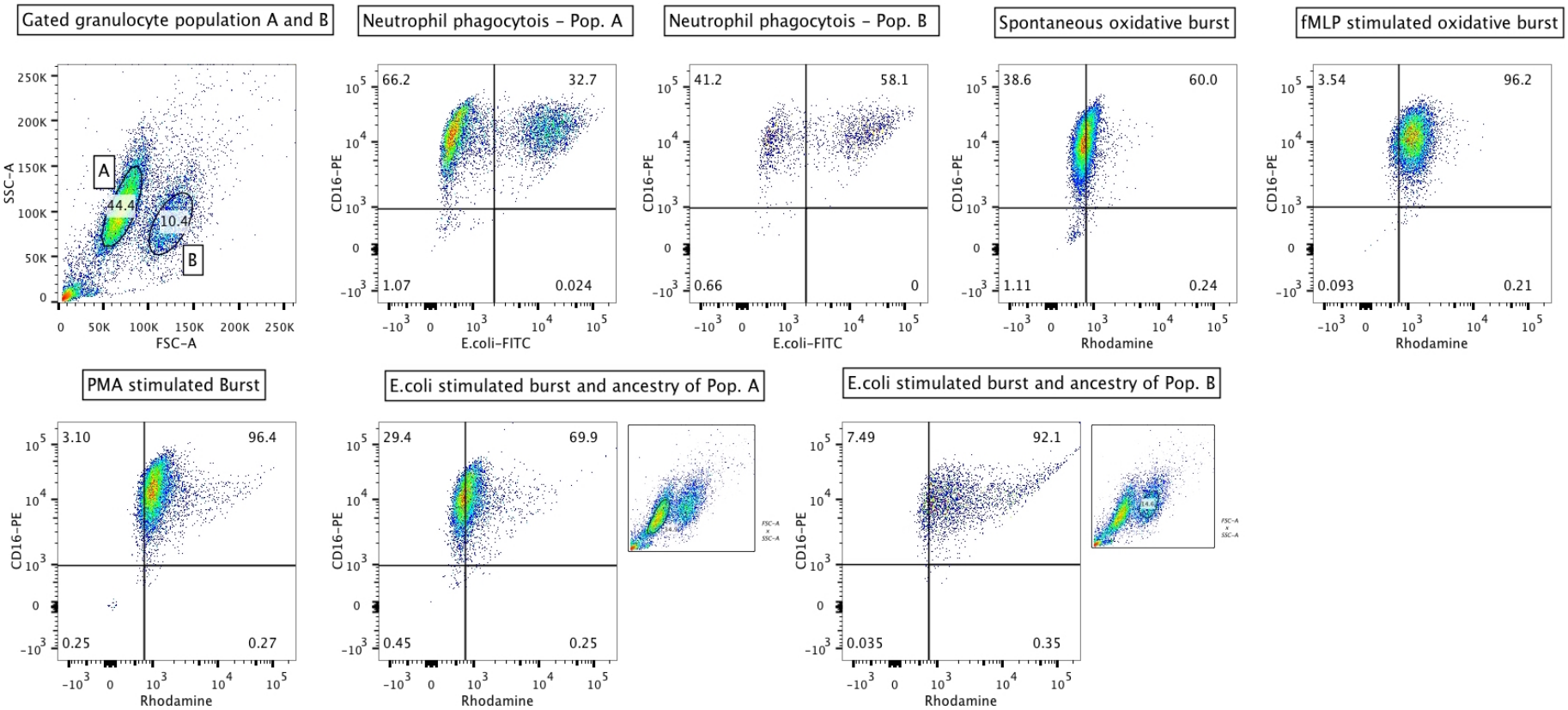


FIGURE 7-4 FLOW CYTOMETER PLOTS OF NEUTROPHIL FUNCTION IN A SEPTIC CONTROL. TWO GATED GRANULOCYTE POPULATIONS ARE EVIDENT CONSISTENT WITH THE DEVELOPMENT OF TOXIC GRANULATION.

Abbreviations: fMLP: Formyl-methionyl-leucyl-phenylalanine; PMA: Phorbol myristate acetate; CD16 PE: Cluster of differentiation 16 – Phycoerytherin; FITC: Fluorescein isothiocyante

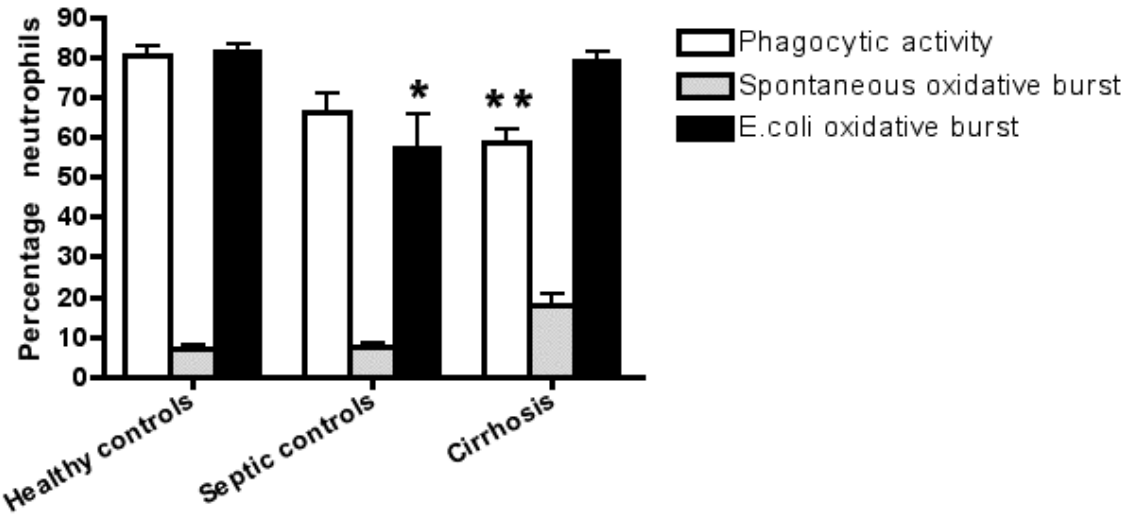


FIGURE 7-5 BASELINE NEUTROPHIL FUNCTION INDICES IN HEALTHY CONTROLS COMPARED TO SEPTIC CONTROLS AND PATIENTS WITH CIRRHOSIS.

Differences between healthy controls (HC) and patients were calculated using the Kruskal-Wallis test with post-hoc analysis using Dunn's multiple comparison test (*$p<0.05$, **$p<0.01$). Neutrophil phagocytic activity was reduced in the cirrhosis cohort ($p=0.002$) compared to HC. Neutrophil *E. coli* stimulated oxidative burst was decreased in the septic control cohort compared to HC ($p<0.05$).

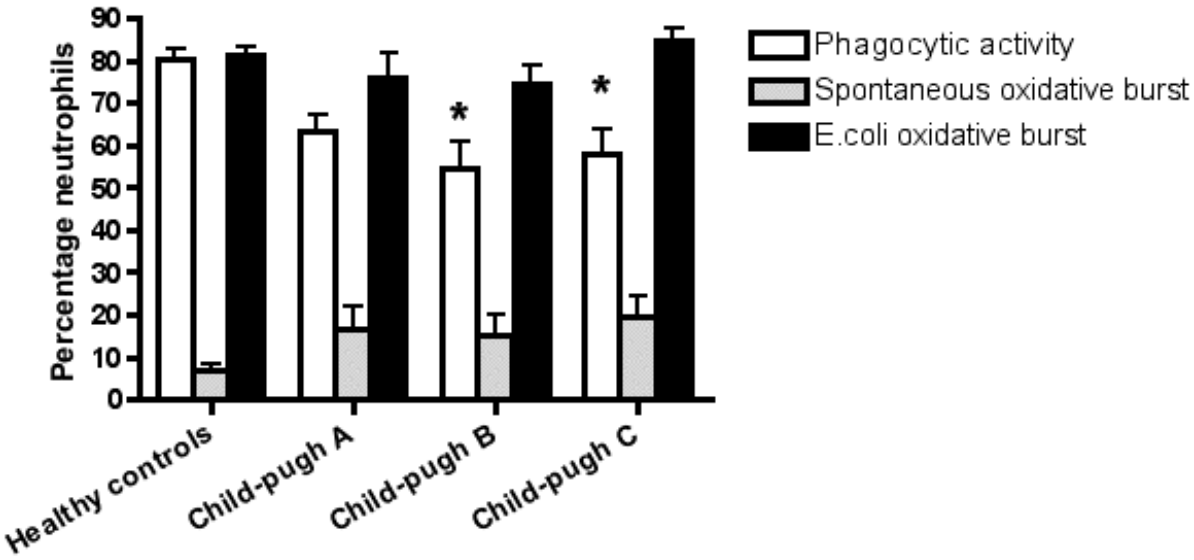


FIGURE 7-6 BASELINE NEUTROPHIL FUNCTION AND SEVERITY OF LIVER DISEASE DETERMINED BY CHILD-PUGH SCORE.

Differences between healthy controls (HC) and patients were calculated using the Kruskal-Wallis test with Dunn's multiple comparison test (*$p<0.05$).

This shows that patients with Child Pugh B (CPB) and C (CPC) cirrhosis had impaired Neutrophil phagocytic activity compared to HC [HC versus CPB; $p=0.026$ and HC versus CPC; $p=0.023$].

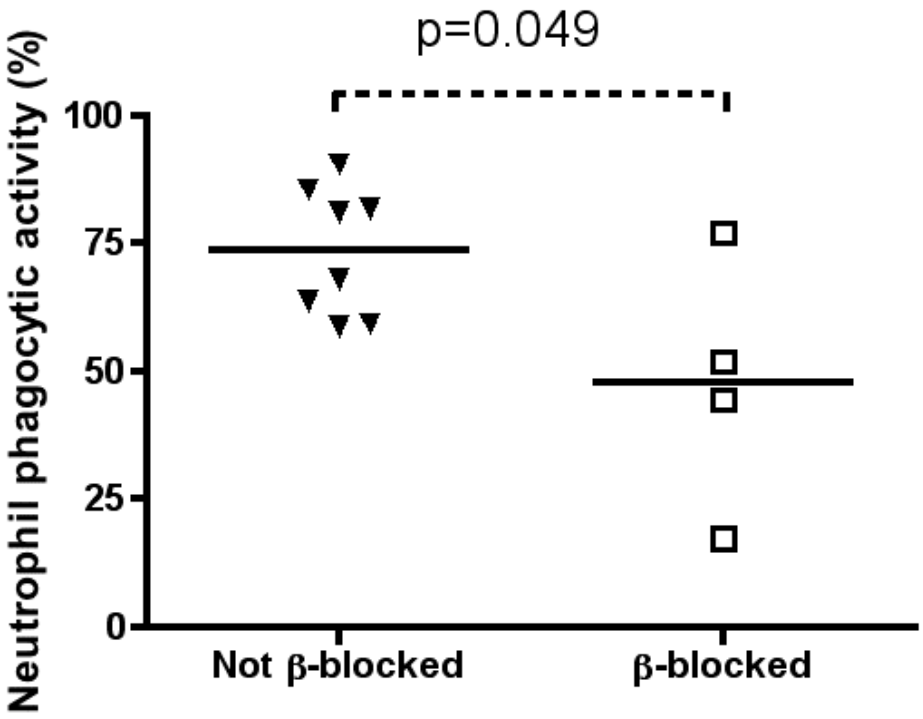


FIGURE 7-7 EFFECT OF NON-SELECTIVE B-BLOCKERS ON NEUTROPHIL PHAGOCYTIC ACTIVITY IN PATIENTS WITH CHILD-PUGH C CIRRHOSIS AND DIURETIC REFRACTORY ASCITES.

Patients on β-blockers had significantly worse NPA (p=0.049) than patients with similar disease severity not treated with β-blockers. Diuretic refractory (intolerant/resistant) ascites was defined as ascites that cannot be mobilized or the early recurrence of which cannot be satisfactorily prevented by medical therapy. (Arroyo and Colmenero, 2003)

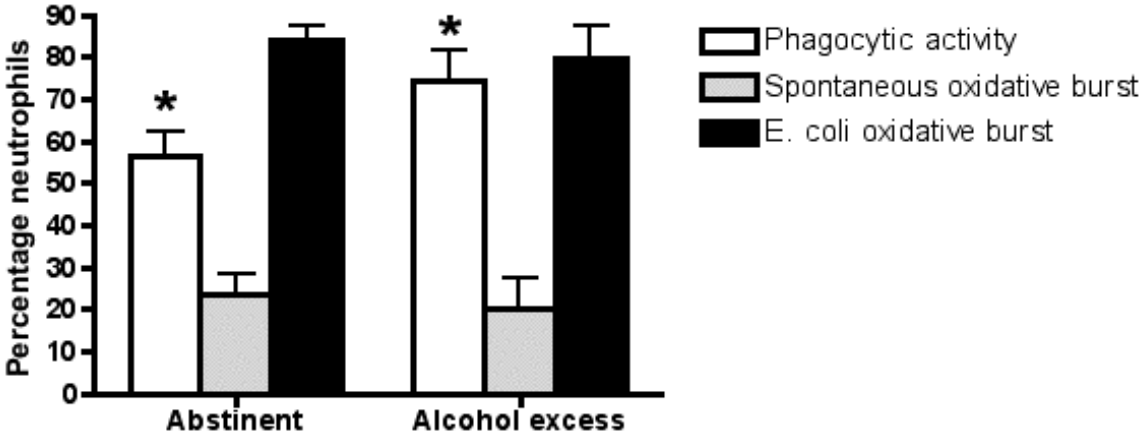


FIGURE 7-8 BASELINE NEUTROPHIL FUNCTION INDICES IN ABSTINENT AND ACTIVE DRINKERS WITH ALCOHOL-RELATED LIVER CIRRHOSIS

Differences between active alcohol drinkers and the abstinents were compared using the Mann-Whitney U test. Neutrophil phagocytic activity was paradoxically higher in the active drinkers compared to the abstinent alcohol drinkers (p=0.029).

Table 7-4 Neutrophil function in abstinent and active drinking patients with cirrhosis due to alcohol-related liver disease (ARLD) †

Neutrophil function	**ARLD cirrhosis abstinent**	**ARLD cirrhosis active drinkers**	**p-values**
Neutrophil Phagocytic Activity (%)	59.9 (46.5-69.4)	79.0 (74.2-83.4)	**0.029**
Neutrophil Resting Burst (%)	26.6 (8.7-35.1)	8.9 (7.5-27.2)	0.940
Neutrophil Stimulated Burst (%)	87.1 (78.8-93.1)	89.2 (64.2-9.5)	0.603

Data presented as median percentage (Interquartile range) of gated neutrophil population undergoing phagocytosis of FITC-labelled E.coli. p-values calculated using the Mann-Whitney U test.

† Stable cirrhosis with no evidence of overt hepatic encephalopathy or organ dysfunction/failure.

7.3.3 Neutrophil oxidative burst (OB)

Neutrophil spontaneous production of ROS (spontaneous oxidative burst [SOB]) was unchanged overall in both patients with cirrhosis and SC compared with HC (p=0.014 and p=0.23 respectively) [Table 7-3 and Figure 7-5]. However, cirrhotic patients displayed a highly variable SOB response with 16/49 (33%) showing an elevated SOB (>12%), compared to none of the HC and only 1/6 (17%) of the SC. SOB was not influenced by CPS (p=0.56) [data not shown]. SOB was unchanged when comparing aetiology, however, intensity of SOB as determined by rhodamine MFI was reduced in patients with autoimmune liver disease compared to other aetiologies of liver disease (p=0.016) [Figure 7-9]. Overall 60% (6/10) of patients with autoimmune cirrhosis were receiving immunomodulatory therapy: 1 patient was receiving azathioprine monotherapy; 2 patients were on combination azathioprine and low dose prednisolone therapy (less than 10mg prednisolone/day); 3 patients were receiving low dose prednisolone monotherapy. An additional 3 patients with autoimmune liver cirrhosis were receiving ursodeoxycholic acid and 1 patient was on no therapy. The intensity of the SOB positively correlated with neutrophil resting and stimulated CD16 expression ($p<0.001$; $r^2=0.63$) and ($p<0.001$; $r^2=0.46$), respectively and resting CD11b expression ($p=0.001$; $r^2=0.55$) [data not shown].

Neutrophil *E.coli* stimulated burst (ESOB) was reduced in SC compared to HC (p=0.034) [Figure 7-5]. The percentage of neutrophils undergoing ESOB in patients with cirrhosis was not increased compared to HC (p=0.147) [Table 7-3 and Figure 7-5]. However, the neutrophils bursting in stable cirrhosis produced significantly more ROS as measured by rhodamine MFI compared to HC (p=0.027) [Table 7-3]. Aetiology of liver disease (p=0.054), CPS (p=0.48) [Figure 7-6] and active alcohol consumption (p=0.603) did not impact on ESOB [Table 7-4].

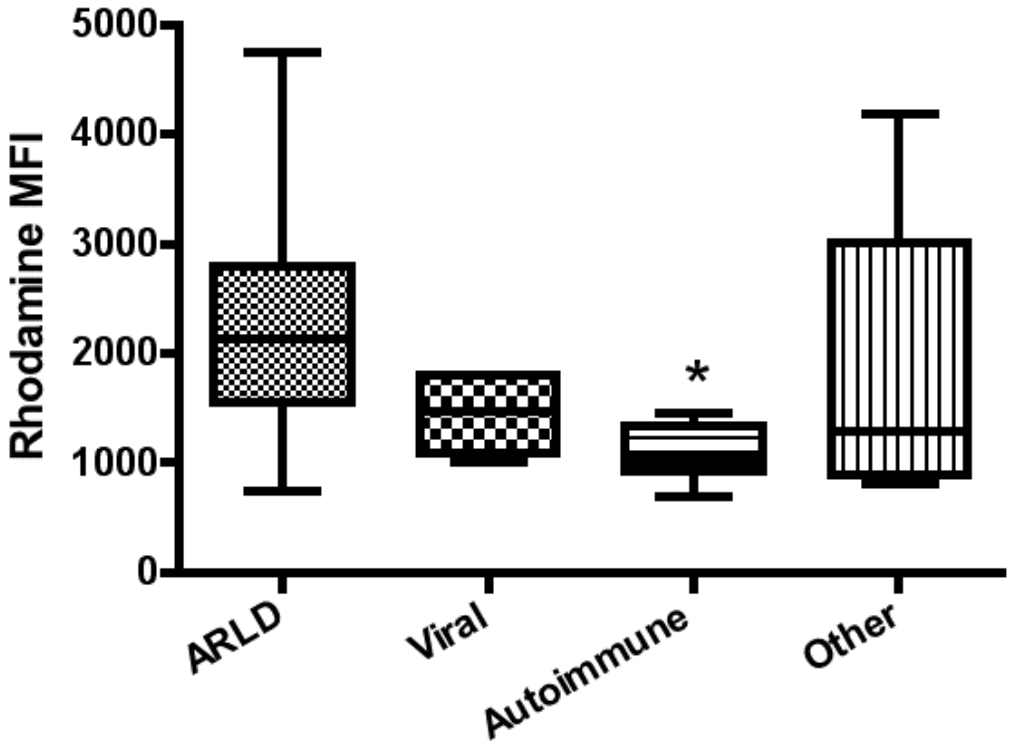


FIGURE 7-9 BOX AND WHISKER PLOT OF NEUTROPHIL SPONTANEOUS RHODAMINE MEAN FLOURESCENCE INTENSITY DEPENDENT ON AETIOLOGY OF LIVER DISEASE.

Differences between aetiology of liver disease were compared using the Mann-Whitney U test, *$p<0.05$.

Abbreviations: ARLD: Alcohol-related liver disease; Autoimmune: Autoimmune liver disease; Viral: Chronic viral hepatitis.

7.3.4 Neutrophil chemotaxis

Neutrophil chemotaxis was examined *ex vivo* in neutrophils from 7 patients with cirrhosis (3 stable cirrhosis; 4 HE; 1 AoCLF), one patient post-LT and two healthy control samples. Patient characteristics are shown in Table 7-5. In a normal neutrophil population the number of neutrophils showing reduced CD62L (L-selectin) expression would be expected to be less than 10-15%. Neutrophil activation is associated with loss of CD62L through proteolytic cleavage by ADAM-17. Increased neutrophil activation as determined by greater than 10% of the unstimulated neutrophil population showing reduced CD62L expression was observed in two patients with cirrhosis at baseline but not in a further cirrhotic. Following fMLP stimulation greater than 90% of neutrophils would be expected to demonstrate reduced CD62L expression. Neutrophil activation of >90% was achieved following fMLP stimulation in all patients with cirrhosis. Neutrophil migration across the cell culture insert was unchanged following fMLP stimulation in 2 patients with cirrhosis and a further sample showed a 7 fold-increase from baseline. The ratio of migrated neutrophils in unstimulated and fMLP stimulated samples ranged from 0.8-7.2 in the patients with cirrhosis and 15.5-26.4 in HC [Table 7-6]. Following LT neutrophil activation (low CD62L expression) at rest returned to <10% but a high level of unstimulated neutrophil migration was seen with an appropriate enhanced neutrophil migration response following fMLP exposure [Table 7-6]. Neutrophil chemotaxis was studied in 4 patients with HE and 1 patient with AoCLF. Unfortunately, a patient sample from one of the HE patients spoiled so baseline CD62L expression could not be determined. However, three patients (2 patients with HE and 1 patient with AoCLF) showed a reduced level of baseline neutrophil CD62L expression and higher levels of baseline migration across the cell culture insert. Neutrophil chemotactic response to fMLP was impaired in patients with HE and AoCLF with migration decreasing in 4 patients (3 x HE and 1 x AoCLF) and one patient with HE mounting only a modest 5-fold increase. The ratio of migrated neutrophils in unstimulated and fMLP stimulated samples ranged from 0.04-4.7 in HE and AoCLF patients compared to 0.4-7.2 in cirrhosis and 15.5-26.4 in HC [Table 7-5].

Table 7-5 Characteristics of cirrhotic patients selected for neutrophil chemotaxis assay.

	Healthy controls (N=2)	**Cirrhosis (N=3)**	**HE (N=4)**	**AoCLF (N=1)**	**Cirrhosis post-LT (n=1)**
Age	29-33	45 (22-65)	42 (29-49)	39	37
Female	0	2	1	0	0
Aetiology		HCV/ARLD/AIH	ARLD	ARLD	PSC
Child-Pugh class (A/B/C)		0/1/2	0/1/3	0/0/1	-
Ascites		2	2	1	-
Albumin (g/L)		38 (25-31)	29 (28-44)	19	23
Bilirubin (μmol/L)		148 (55-369)	162 (133-350)	118	19
WCC (x 10^9/L)		7.2 (3.8-8.3)	11.0 (2.4-12.2)	18.6	5.5
CRP (mg/L)		51 (7-64)	29 (27-30)	123	29
AST (IU/L)		122 (54-123)	75 (66-94)	71	27
Antibiotics		2	3	1	0

Data presented as median (range) as appropriate.

Abbreviations: AoCLF: Acute-on-chronic liver failure; AIH: autoimmune hepatitis; AST: Aspartate Aminotransferase; ARLD: alcohol-related liver disease; CRP: C-reactive protein; HCV: Hepatitis C virus; HE: Hepatic encephalopathy; LT: liver transplantation; PSC: primary sclerosing cholangitis; WCC: white cell count.

Table 7-6 Neutrophil migration through cell culture inserts with a pore size of 3.0 μm towards a concentration gradient of fMLP.

Sample	Unstimulated neutrophil migration	Control activated neutrophils (%)	fMLP stimulated neutrophil migration	fMLP activated neutrophils (%)	Ratio of Unstimulated : fMLP stimulated neutrophils	Plasma Ammonia μmol/L
HC1	92	10	1427	93	15.5	
HC2	578	6	15261	95	26.4	
Cirrhosis1	106	21	82	92	0.8	79
Cirrhosis2	5565	6	5157	99	0.9	94
Cirrhosis3	239	14	1723	94	7.2	52
HE1	525	9	57	95	0.13	112
HE2	5588	24	325	85	0.66	53
HE3	4716	25	177	97	0.04	186
HE4	55		263	92	4.78	41
AoCLF1	3650	6	175	97	0.05	70
Cirrhosis Post-LT	4507	9	20279	97	4.5	20
ALF	3576	26	5514	99	1.54	40

Absolute number of migrated neutrophils acquired by flow-cytometry after acquisition of 2000 counting bead events. Shaded cells indicate results not available.
Abbreviations: ALF: acute liver failure; AoCLF: Acute-on-chronic liver failure; Formyl-methionyl-leucyl-phenylalanine; HC: Healthy control; HE: Hepatic encephalopathy: LT; liver transplantation.

7.3.5 Relationship between neutrophil function, plasma ammonia, serum sodium and biochemical parameters

In all patients with cirrhosis, elevated plasma ammonia concentration correlated with decreasing NPA ($p=0.01$; $r^2=0.13$) [Figure 7-10]. Decreasing NPA was also associated with declining neutrophil count ($p<0.001$; $r^2=0.185$) and increasing IgG levels ($p=0.041$; $r^2=0.185$) [Figure 7-15]. NPA was not influenced by serum sodium ($p=0.98$), serum CRP levels ($p=0.054$ or concomitant antibiotic prescription ($p=0.217$). Other laboratory markers of inflammation and organ function as stated in Table 7-2 were compared to NPA but no association was found.

Baseline clinical and laboratory parameters did not show any association with SOB or ESOB in patients with cirrhosis.

7.3.6 Relationship between neutrophil function and plasma cytokines

Plasma concentrations of pro- and anti-inflammatory cytokines were increased in patients with stable cirrhosis compared to levels seen in HC when comparing the natural logarithm (ln) of levels of TNFα, IL-1β, IL-6, IL-8, IL-10 and IL-17, $p<0.01$ for all comparisons) [Table 7-3]. Cytokine levels were also elevated in cirrhotic patients compared to SC. Only levels of IL-6 and IL-8 in SC were significantly elevated above levels seen in HC (both $p<0.05$) suggesting that the intensity of the inflammatory response was greatest in the cirrhotic cohort. Cytokine levels were elevated in a similar manner across all 3 Child-Pugh classes (A-C) suggesting the inflammatory response is independent of liver disease severity [data not shown].

Declining NPA correlated with increasing plasma IL-17 concentration ng/ml ($p=0.031$:$r^2$0.122) [Figure 7-11].

Increased SOB correlated with increasing levels of lnTNFα ($p=0.012$; $r^2=0.13$), lnIL-6 ($p=0.006$; $r^2=0.15$), lnIL-8 ($p=0.004$; $r^2=017$) and lnIL-10 ($p=0.018$; $r^2=0.115$) [Figure 7-12]. Increased ESOB correlated with increasing levels of lnIL-1β ($p=0.021$; $r^2=0.13$), InIL-8 ($p=0.002$; $r^2=0.2$), InIL-10 ($p=0.003$; $r^2=0.17$) and InIL-17 ($p=0.005$; $r^2=0.15$) [Figure 7-13].

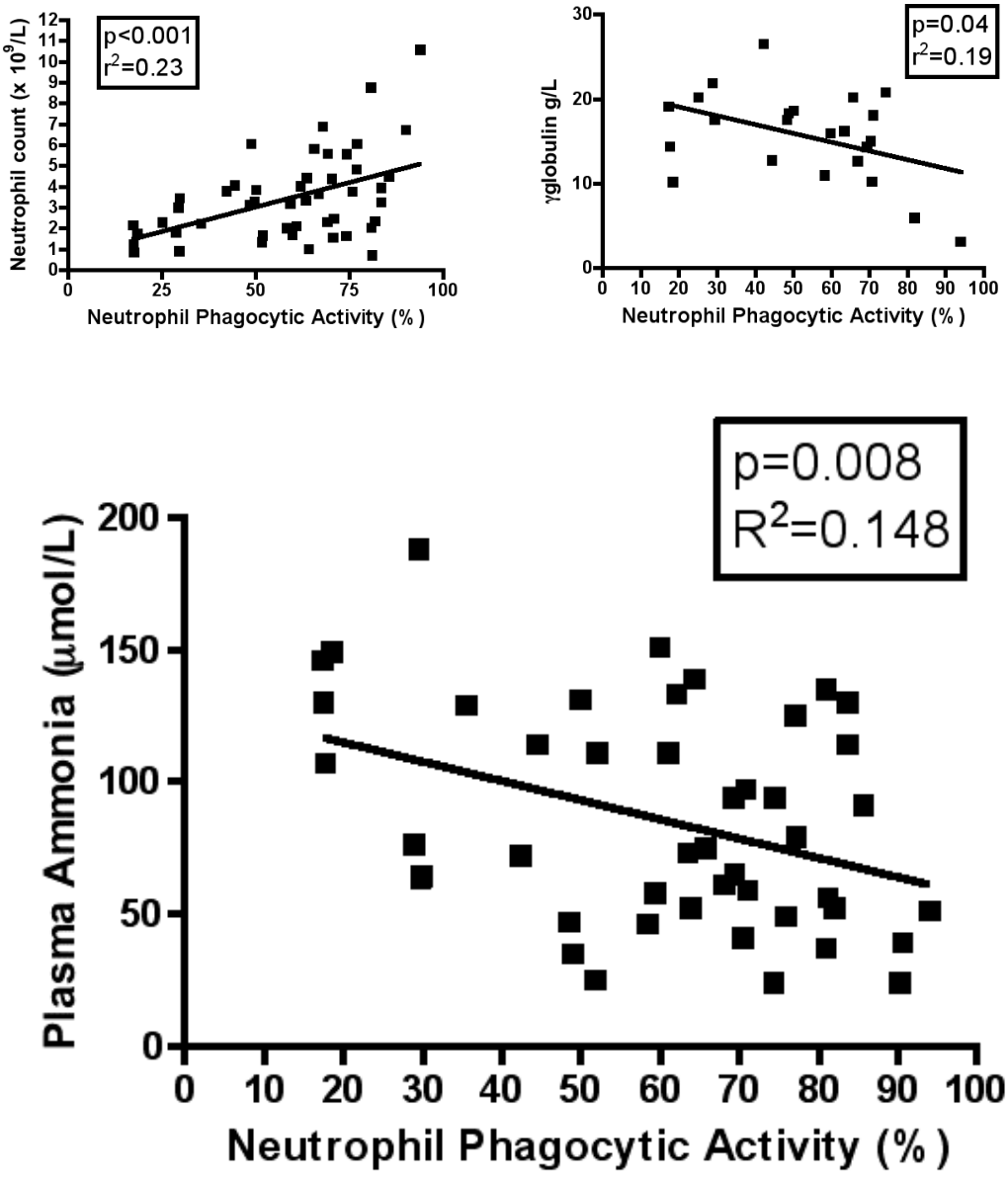


FIGURE 7-10 ASSOCIATIONS BETWEEN NEUTROPHIL PHAGOCYTIC ACTIVITY AND NEUTROPHIL COUNT, GAMMA GLOBULIN AND PLASMA AMMONIA IN PATIENTS WITH STABLE CIRRHOSIS.

7.3.7

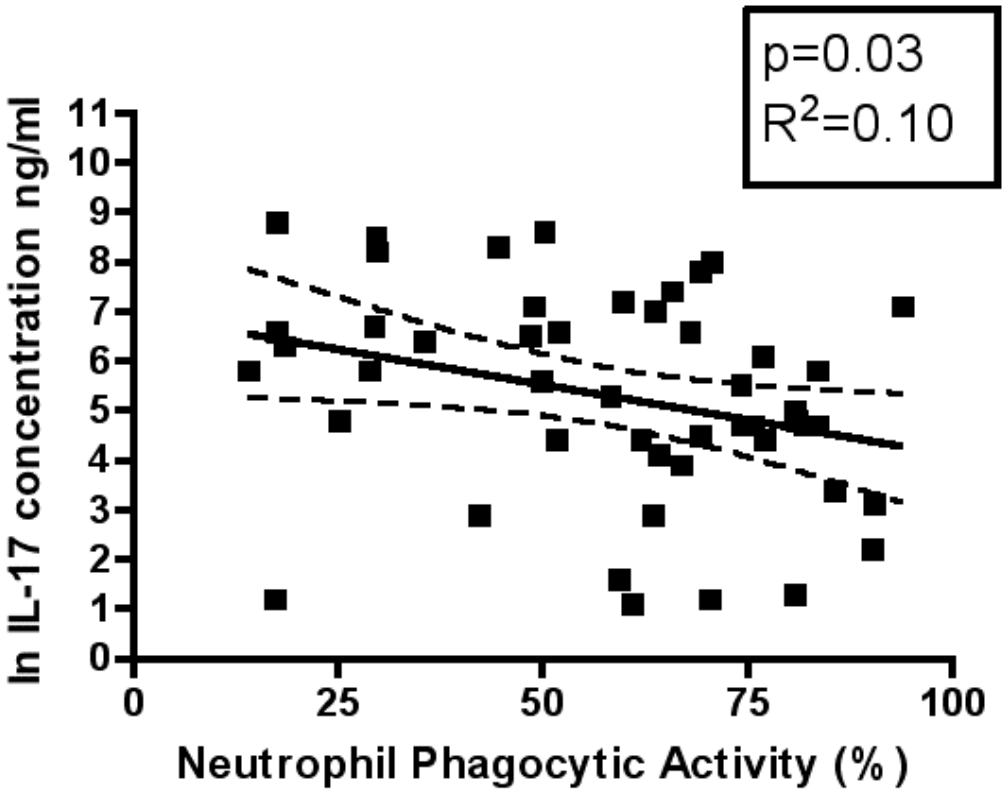


FIGURE 7-11 LOGISTIC REGRESSION ANALYSIS OF NEUTROPHIL PHAGOCYTIC ACTIVITY AND THE NATURAL LOGARITHM OF IL-17 CONCENTRATION NG/ML IN PATIENTS WITH STABLE CIRRHOSIS.

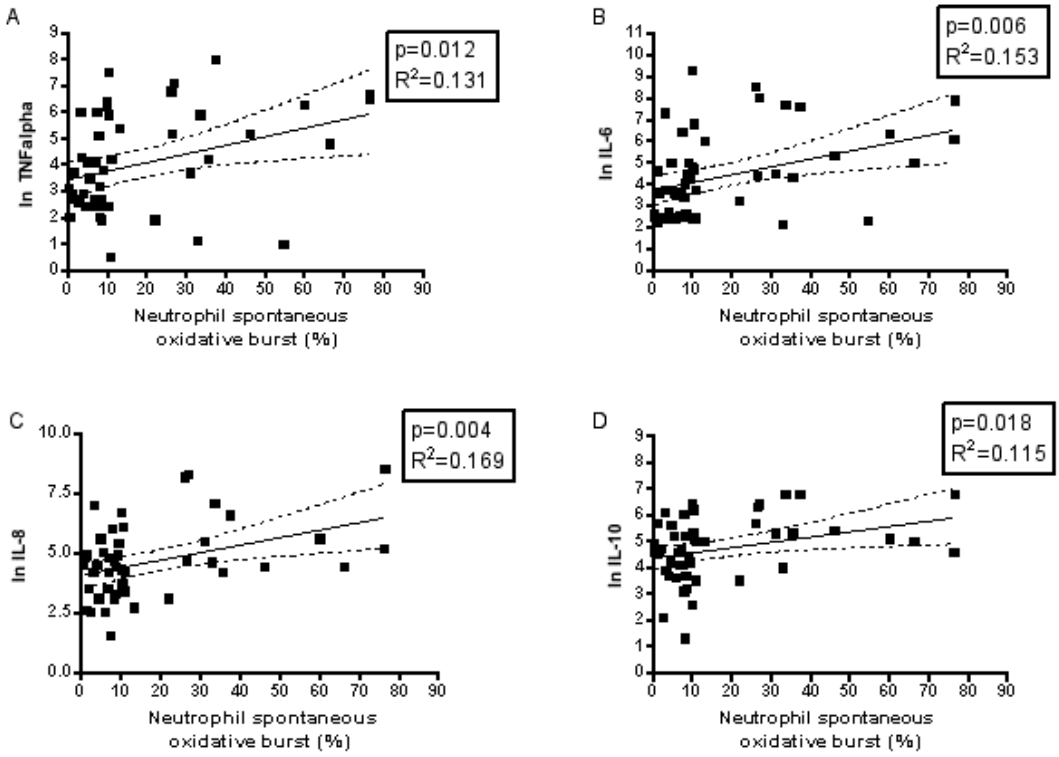


FIGURE 7-12 LOGISTIC REGRESSION ANALYSIS OF NEUTROPHIL SPONTANEOUS OXIDATIVE BURST AND PLASMA LEVELS OF PRO- / ANTI-INFLAMMATORY CYTOKINES IN PATIENTS WITH STABLE CIRRHOSIS.

Increasing neutrophil SOB was associated with elevated levels of TNFα, IL-6, IL-8 and IL-10.

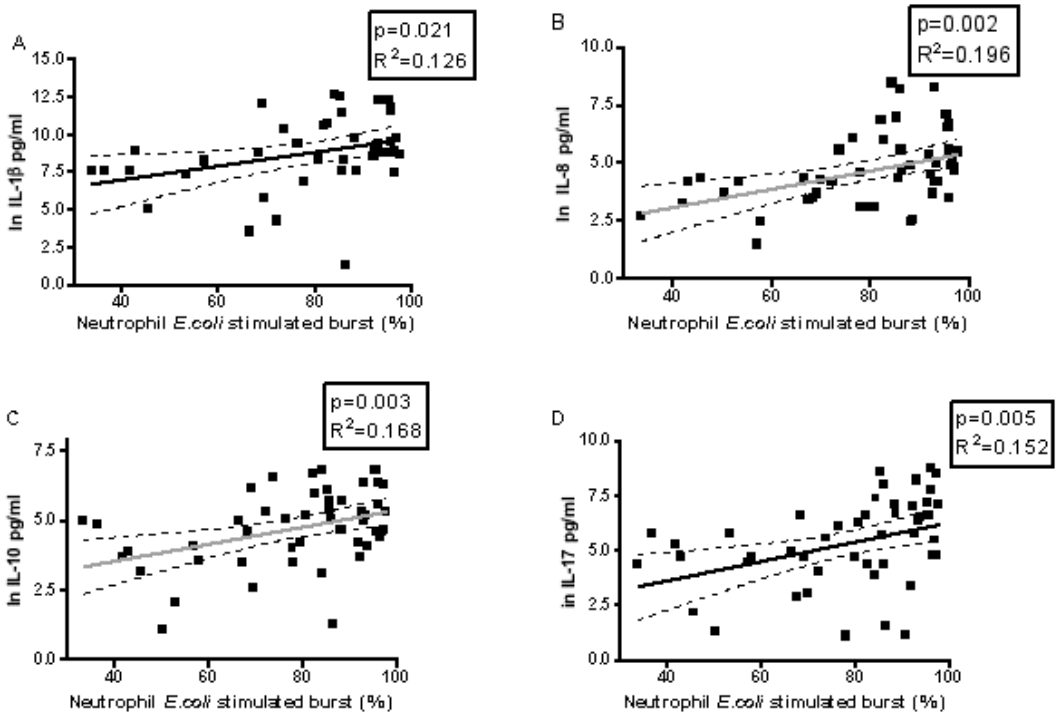


FIGURE 7-13 LOGISTIC REGRESSION ANALYSIS OF NEUTROPHIL *E.COLI* STIMULATED OXIDATIVE BURST AND PLASMA LEVELS OF PRO- / ANTI-INFLAMMATORY CYTOKINES IN PATIENTS WITH STABLE CIRRHOSIS.

Increasing neutrophil ESOB was associated with elevated levels IL-1β, IL-8, IL-10 and IL-17.

7.4 Study to determine the contribution of neutrophil dysfunction on the increased susceptibility to infection seen in patients with cirrhosis

The number of patients undergoing LT, and 90-day and 1-year LT-free survival are shown in Table 7-3 and Figure 7-1. LT-free survival was significantly higher in the stable cirrhosis cohort compared to the AoCLF cohort both at 90-days, and at 1-year ($p<0.01$).

Seven deaths (14%) occurred in the 12 months following recruitment in the stable cirrhosis cohort (5 deaths occurred within 90-days). Two patients developed MODS secondary to infection; 1 polymicrobial infection including candidaemia (endocarditis); and 1 bowel perforation. Five died following discharge (4 had been declined LT due to co-morbidity and 1 after alcohol recidivism). Median time to death was 77-days (range 46-196). Sixteen patients in the cirrhosis cohort underwent successful LT after a median of 34-days (range 5-316). One patient developed HE during the same admission and underwent LT after 22 days. Six further episodes of infection occurred within 90-days [3 bacteraemias, 1 urinary tract infection and 2 SBP].

In the AoCLF group eight deaths occurred at a median of 12 days (range 1-81). Seven deaths occurred due to MODS secondary to bacterial/fungal bacteraemia. One patient developed peritonitis due to a diverticular perforation and was managed with surgical resection and survived to discharge after a 10-day ICU stay. Three of the AoCLF cohort underwent successful LT after 1, 3 and 45-days (3 died on the waiting list).

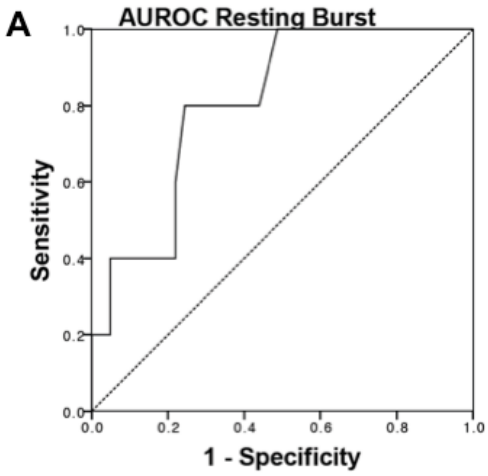
Overall in all the cirrhotic patients recruited, 26% (n=19) either had infection at the time of sampling (AoCLF) or developed an episode in the subsequent 90-days.

Neutrophil function indices were good predictors of outcome and survival in both stable cirrhosis and AoCLF. Neutrophil SOB on recruitment was a predictor of survival at 90-days and 1-year from baseline on ROC curve analysis AUROC 0.81 (95% CI 0.64-0.97); p=0.026 and 0.76 (95% CI 0.61-0.91); p=0.03 respectively [Figure 7-14]. Using SOB ≥12% as a cut-off predicted 90-day mortality with 80% sensitivity and 71% specificity. Furthermore, evaluating 90-day survival using a Kaplan-Meier and log rank analysis, SOB ≥12% differentiated survivors from non survivors (p=0.015) [Figure 7-14] but just failed to reach significance at 1-year (p=0.053).

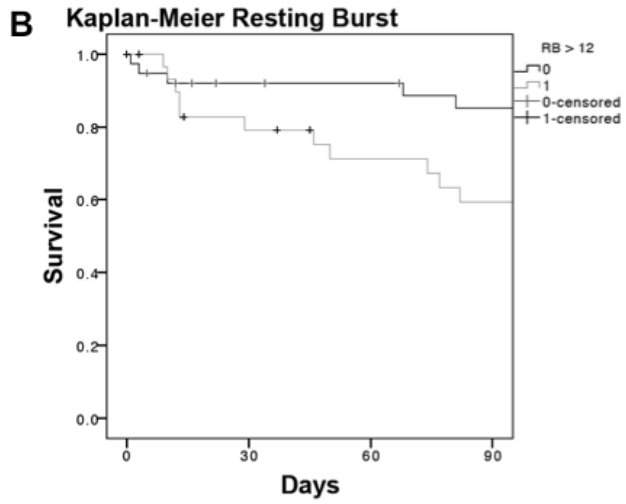
Neutrophil phagocytic capacity (NPC), as determined by the FITC MFI, predicted 90-day survival in the cirrhosis cohort. On ROC curve analysis AUROC 0.83 (95% CI 0.68-0.97); p=0.021 and using NPC ≥ 19167 as a cut-off predicted 90-day

survival with 78% sensitivity and 80% specificity; p=0.01 on Kaplan-Meier log-rank analysis and 1-year survival p<0.001 [Figure 7.15].

NPA predicted a composite end-point of active infection and subsequent development of infection within 90-days of sampling on ROC curve analysis AUROC 0.71 (95% CI 0.56-0.86); p=0.011. Using NPA<67.2% as a cut-off was predictive of active/subsequent infection with 72% sensitivity and 70% specificity [data not shown].



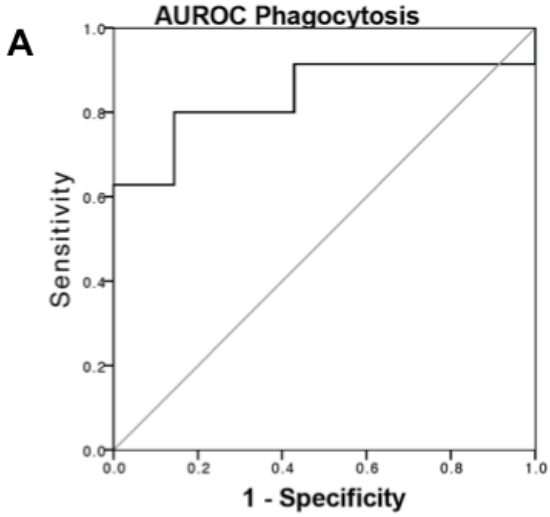
Area	Std. Error	Significance	Cut-off	Sensitivity	Specificity
0.81	0.085	0.026	12	0.80	0.71



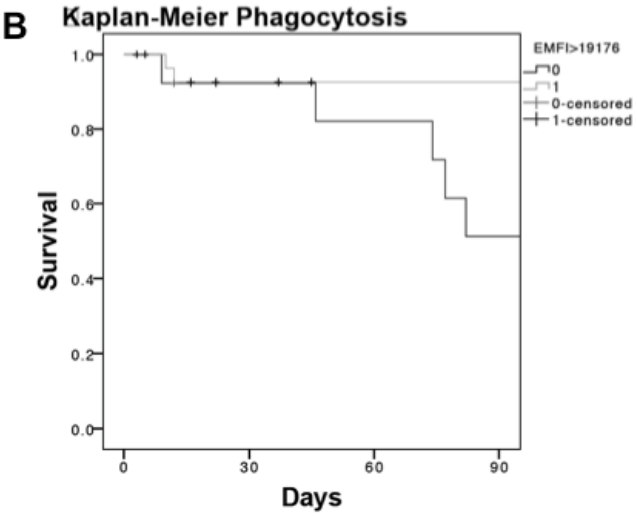
Day	30	60	90
Events	10	11	16
At risk	69	60	57

FIGURE 7-14 AREA UNDER THE RECEIVER OPERATOR CURVES [AUROC] (A) AND KAPLAN-MEIER SURVIVAL ANALYSES (B) AS DEFINED BY ADMISSION NEUTROPHIL RESTING OXIDATIVE BURST IN PATIENTS WITH STABLE CIRRHOSIS.

SOB was a predictor of poor outcome with an AUROC of 0.81 (95% CI 0.68-0.97) and using neutrophil SOB>12% as a cut-off gives a sensitivity of 80% and specificity of 71% with an increased 90-day mortality demonstrated on Kaplan-Meier log-rank analysis (p=0.015).



Area	Std. Error	Significance	Cut-off	Sensitivity	Specificity
0.83	0.074	0.021	19167	0.78	0.80



Day	30	60	90
Events	3	4	9
At risk	42	35	33

FIGURE 7-15 AREA UNDER THE RECEIVER OPERATOR CURVE [AUROC] (A) AND KAPLAN-MEIER SURVIVAL ANALYSIS (B) AS DEFINED BY ADMISSION NEUTROPHIL PHAGOCYTIC CAPACITY [NPC] IN PATIENTS WITH STABLE CIRRHOSIS.

NPC was a predictor of poor outcome with AUROC of 0.84 (95% CI 0.68-0.97) and using NPC < 19,176 as a cut-off gives a sensitivity of 80% and specificity of 86% with a corresponding increased 90-day mortality demonstrated on Kaplan-Meier log-rank analysis (p=0.01).

7.5 Study to determine the impact of developing hepatic encephalopathy on neutrophil function in cirrhosis

Patients with HE showed a number of differences in their baseline characteristics when compared to the stable cirrhosis cohort [Tables 7-1 and 7-2]. In the HE cohort the predominant aetiology of liver disease was ARLD (69%), 77% of patients had low grade HE (West-Haven I-II) and 23% had high grade (West-Haven III-IV). (Harold and Milton, 1979) The majority were receiving empirical antibiotics (23% had active infection) and 90-day transplant free survival was inferior when compared to patients with stable cirrhosis (45% v's 69%, $p<0.05$). When comparing the HE cohort to patients with stable cirrhosis baseline laboratory parameters of inflammation, renal function and serum sodium were similar, however, patients with HE had an increased baseline INR compared to patients with stable cirrhosis, $p<0.05$, and as a consequence liver failure scores were increased; CPS ($p<0.01$); MELD ($p<0.05$); and UKELD ($p<0.05$) [Table 7-2]. Interestingly, no difference in plasma arterial ammonia concentration was observed between patients with stable cirrhosis and those with HE ($p=0.786$).

Ongoing impairment of neutrophil phagocytosis was observed following the development of HE with impaired NPA compared to HC ($p=0.005$). [Figure 7-16 and 7-17 and Table 7-7]. There was enhanced spontaneous production of ROS (SOB) from neutrophils in HE with elevated SOB compared to patients with stable cirrhosis ($p=0.032$) [Figures 7-16 and 7-17]. ESOB was unchanged in HE compared to HC and stable cirrhosis. In the HE cohort an association was observed between increasing levels of bilirubin and deteriorating NPA ($p=0.017$, $r^2=0.45$) [Figure 7-19]. Increased neutrophil SOB was associated with increasing neutrophil cell volume assessed by neutrophil forward scatter Geometric MFI ($p=0.012$, $r^2=0.69$) [Figure 7-19].

7.6 Study to determine the impact of developing acute-on-chronic liver failure on neutrophil function in cirrhosis

In the AoCLF cohort the aetiology of liver disease was predominantly ARLD or autoimmune liver diseases. The majority of AoCLF patients had DRA and were receiving antibiotics (92%) with active infection being seen in 58%. The 90-day transplant free survival was significantly inferior to patients with stable cirrhosis (8% versus 69%, $p<0.001$). Patients with AoCLF exhibited significant increases in

inflammatory markers (neutrophil count and CRP both $p<0.01$); hepatic function (serum bilirubin, INR, AST, all $p<0.01$); and renal function (serum creatinine, $p<0.01$) compared to stable cirrhosis [Table 7-2]. Consequentially patients with AoCLF demonstrated higher baseline liver failure (CPS, MELD and UKELD, all $P<0.01$) and multi-organ failure scores (APACHEII and SOFA, both $P<0.01$) [Table 7-2]. No difference in plasma arterial ammonia concentration was observed between patients with stable cirrhosis and those with AoCLF ($p=0.786$).

Ongoing impairment of neutrophil phagocytosis was seen following the development of AoCLF with impaired NPA compared to HC ($p=0.017$) [Figure 7-16 and 7-17 and Table 7-7]. There was enhanced spontaneous production of ROS (SOB) from neutrophils in AoCLF with elevated SOB compared to patients with stable cirrhosis ($p=0.037$) [Figures 7-16 and 7-17]. *Ex-vivo* neutrophils retained the capacity to generate an oxidative burst after *E.coli* challenge following the development of AoCLF. The trajectory of neutrophil phagocytic dysfunction in patients with stable cirrhosis following the development in infection and subsequent deterioration to AoCLF, was one of a progressive decline over time culminating in profound neutrophil malfunction. This predisposed the patient to secondary infection, MODS and invariably a terminal decline [Figure 7-18].

In patients with AoCLF no correlation was observed between NPA, neutrophil oxidative burst and plasma ammonia concentration. However, reduced NPA was associated with increasing liver transaminases (AST $p=0.01$; $r^2=0.48$) and APACHE II score ($p=0.03$, $r^2=0.32$) [Figure 7-20].

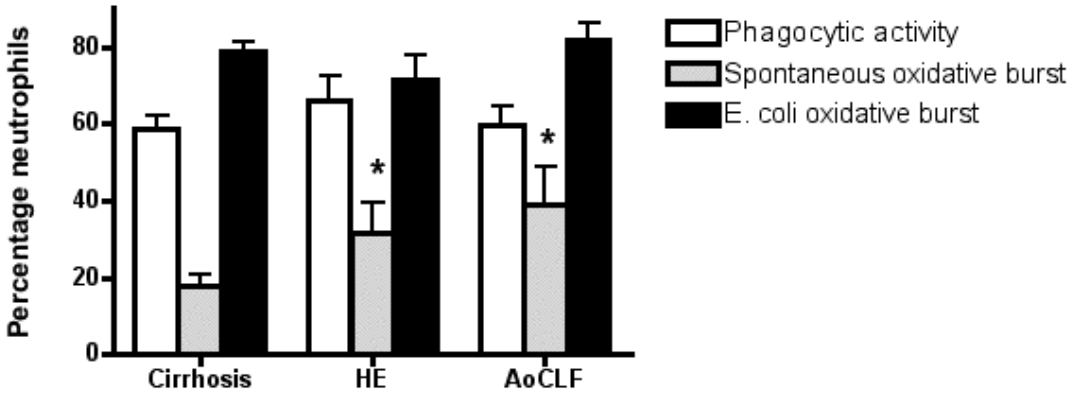


FIGURE 7-16 NEUTROPHIL FUNCTION IN PATIENTS WITH STABLE CIRRHOSIS COMPARED TO PATIENTS WITH CIRRHOSIS FOLLOWING THE DEVELOPMENT OF HEPATIC ENCEPHALOPATHY (HE) AND ACUTE-ON-CHRONIC LIVER FAILURE (AOCLF).

Groups were compared using the Kruskal-Wallis test with Dunn's multiple comparison test, *$p<0.05$. Patients with HE and AoCLF had an enhanced resting production of reactive oxygen species compared to patients with stable cirrhosis ($p=0.032$ and $p=0.037$ respectively).

Table 7-7 Neutrophil function in healthy controls and patients with cirrhosis complicated by hepatic encephalopathy and acute-on-chronic liver failure.

Neutrophil function / plasma cytokine concentration	Healthy controls	Hepatic encephalopathy	Acute-on-chronic liver failure	p-values
Neutrophil phagocytic activity	77.1 (72.2-83.6)	76.3 (57.2-80.1)	66.1 (57.9-72.4)	**0.019***
Spontaneous oxidative burst	8.7 (5.6-9.3)	23.5 (10.7-45.0)	30.5 (8.7-69.2)	**0.014***
E.coli stimulated oxidative burst	84.9 (79.7-85.6)	72.4 (53.2-92.4)	93.7 (79.6-95.7)	0.103
Tumour Necrosis Factor-α pg/mL	9 (3-39)	17 (11-148)	65 (41-154)	0.059
Interlukin-1β pg/mL	4545 (1429-7346)	1481 (1098-6340)	2780 (1125-27116)	0.588
Interleukin -6 pg/mL	21 (16-38)	50 (29-70)	77 (51-213)	**0.016***
Interleukin -8 pg/mL	23 (13-34)	94 (65-128)	217 (160-480)	**<0.001****
Interleukin -10 pg/mL	72 (28-76)	61 (36-153)	197 (71-561)	0.052
Interleukin -17 pg/mL	66 (46-163)	66 (10-131)	174 (81-625)	**0.041***

Data presented as median percentage (inter quartile range) of gated neutrophil population undergoing phagocytosis of FITC-labelled *E.coli*. p-values calculated using the Kruskal-Wallis test with Dunn's multiple comparison test.*= $p<0.05$, **$p<0.01$.

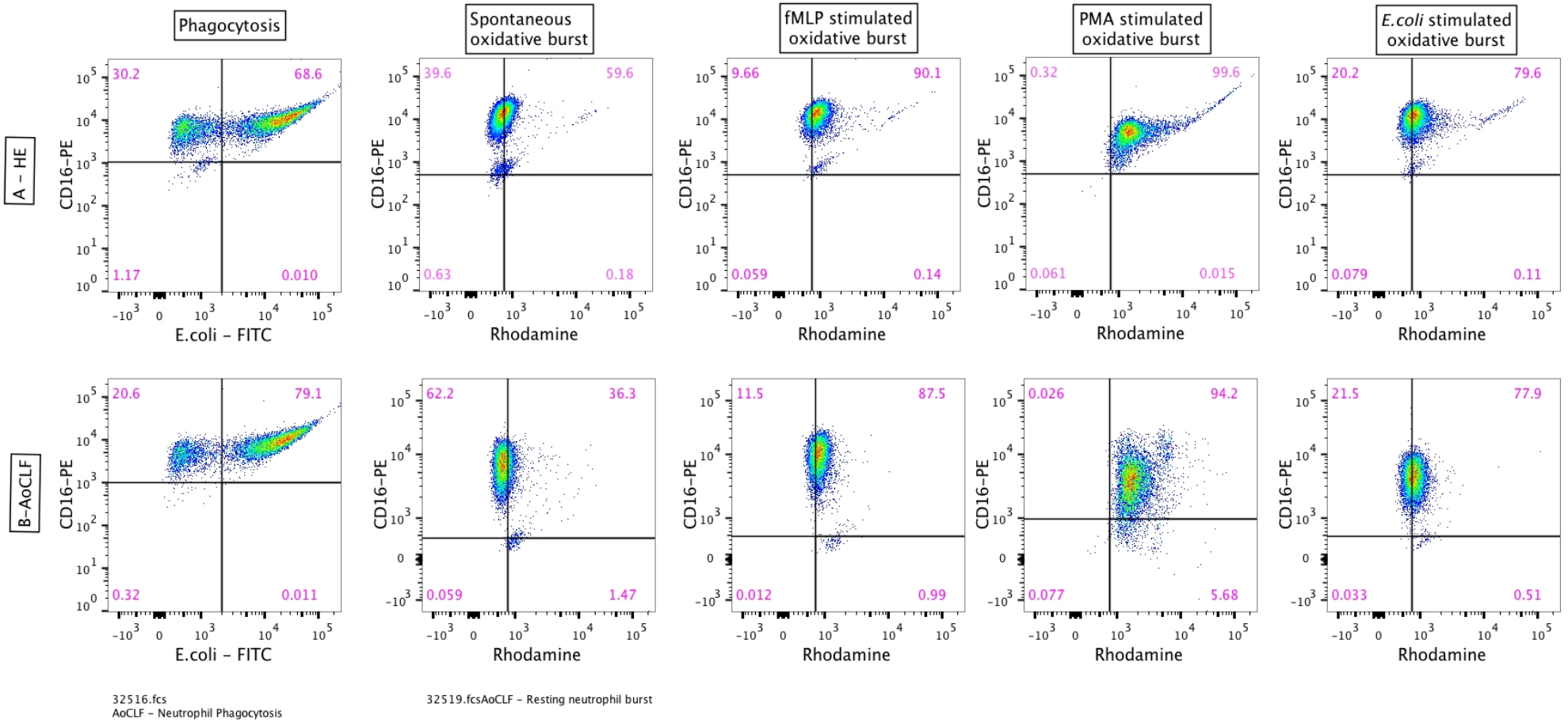


FIGURE 7-17 FLOW CYTOMETER PLOTS OF NEUTROPHIL FUNCTION INDICES IN A PATIENT WITH HEPATIC ENCEPHALOPATHY AND A PATIENT WITH ACUTE-ON-CHRONIC LIVER FAILURE

Abbreviations: fMLP: Formyl-methionyl-leucyl-phenylalanine; PMA: Phorbol myristate acetate; CD16 PE: Cluster of differentiation 16 – Phycoerytherin; FITC: Fluorescein isothiocyanate.

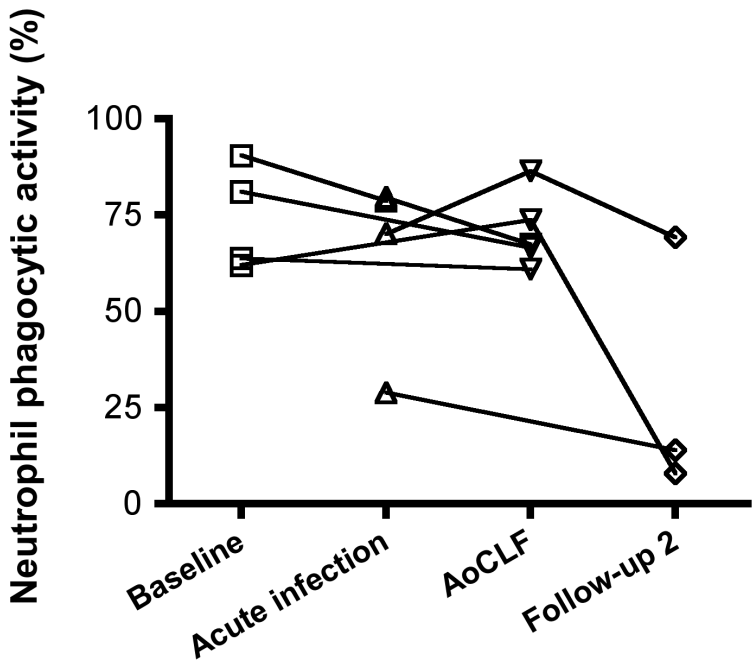


FIGURE 7-18 DYNAMICS OF NEUTROPHIL PHAGOCYTIC ACTIVITY (%) COMPARED TO BASELINE FOLLOWING THE DEVELOPMENT OF INFECTION AND/OR ACUTE-ON-CHRONIC LIVER FAILURE

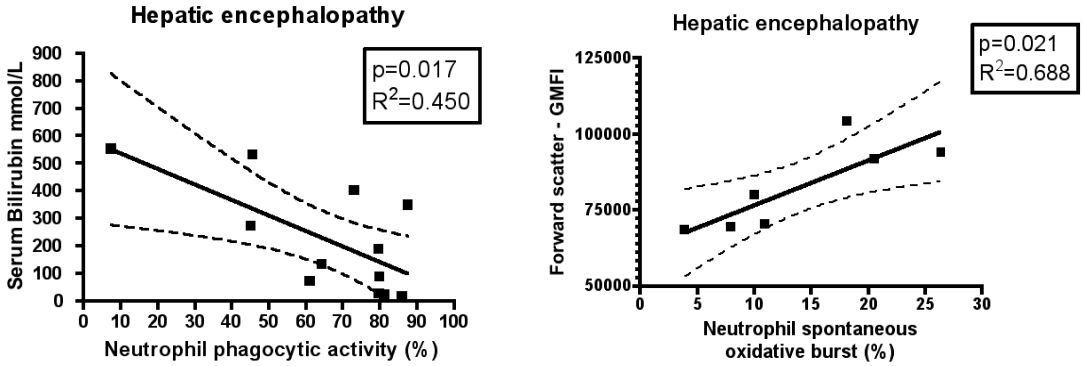


FIGURE 7-19 LOGISTIC REGRESSION ANALYSIS OF NEUTROPHIL PHAGOCYTIC ACTIVITY VERSUS SERUM BILIRUBIN CONCENTRATION IN PATIENTS WITH HEPATIC ENCEPHALOPATHY.

Graph shows association between declining NPA versus increasing serum bilirubin concentration (p=0.017) in patients with hepatic encephalopathy.

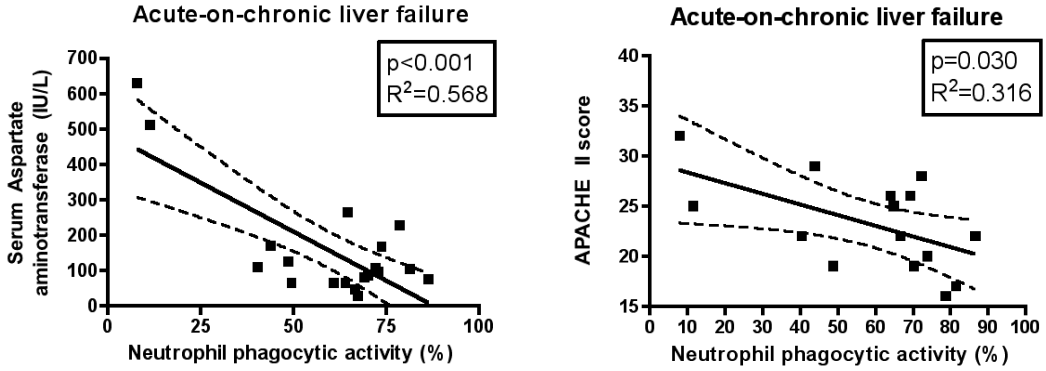


FIGURE 7-20 LOGISTIC REGRESSION ANALYSIS OF NEUTROPHIL PHAGOCYTIC ACTIVITY VERSUS SERUM ASPARTATE AMINOTRANSFERASE AND APACHE II SCORE IN PATIENTS WITH ACUTE-ON-CHRONIC LIVER FAILURE.

Graph shows association between declining NPA and increasing serum AST concentration (p=0.03) and APACHE II score (p=0.01) in patients with acute-on-chronic liver failure.

7.7 Impact of liver transplantation on neutrophil function in cirrhosis

In the cirrhosis cohort 16 patients underwent LT after a median of 34 days (range 5-315). Pre-LT the median plasma ammonia concentration was 70 μmol/L (IQR 52-108) and this dropped to a median of 25 μmol/L (IQR 8-10) 72-hours post-LT ($p<0.01$). Patients were assessed at a median of 9.5 days post LT (IQR 5.8-14.5). However, at the time of assessment serum levels of cytokines TNFα, IL-1β, IL-6, IL-10 and IL-17 were unchanged compared to baseline with only levels of IL-8 showing a significant reduction [data not shown]. There was a tendency towards improvement in NPA and resting and *E. coli* stimulated ROS production in *ex vivo* neutrophils 72-hours post-LT [Figures 7-21 and 7-22]. The response was highly variable with some patients showing a marked improvement especially in those with the most severe impairment, whilst the converse was true in patients who had normal NPA pre-LT. Overall NPA change post-LT failed to reach significance using the Wilcoxon matched pairs test suggesting ongoing impairment in neutrophil phagocytic function possibly driven by the persisting inflammatory response probably as a consequence of major surgical insult and hepatic reperfusion injury independent of plasma ammonia levels.

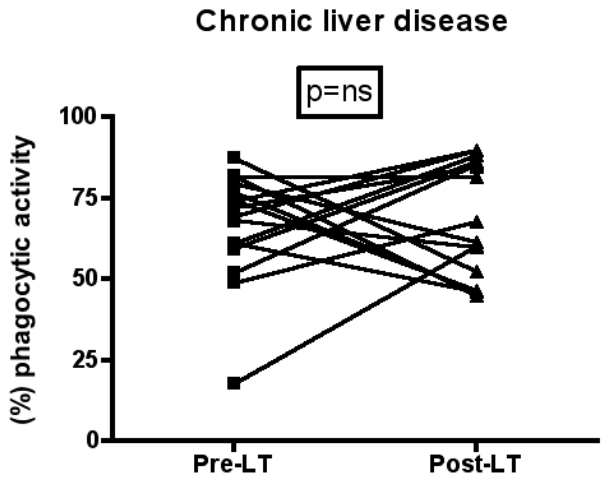


FIGURE 7-21 CHANGES IN *EX VIVO* NEUTROPHIL PHAGOCYTIC ACTIVITY FOLLOWING LIVER TRANSPLANTATION IN PATIENTS WITH CIRRHOSIS. P=NON-SIGNIFICANT USING WILCOXON MATCHED PAIRS TEST.

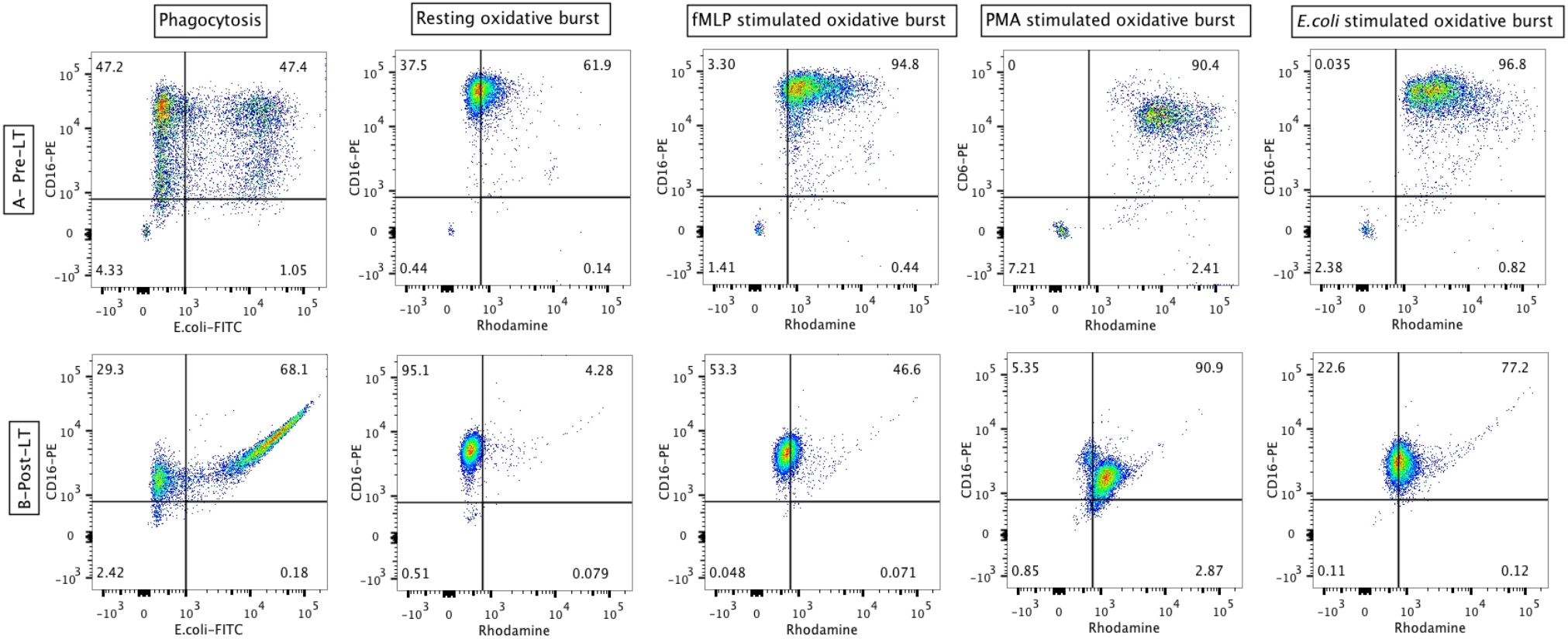


FIGURE 7-22 FLOW CYTOMETER PLOTS OF NEUTROPHIL FUNCTION INDICES OF A PATIENT WITH CIRRHOSIS BEFORE AND FOLLOWING SUCESSFUL LIVER TRANSPLANTATION.

Abbreviations: fMLP: Formyl-methionyl-leucyl-phenylalanine; PMA: Phorbol myristate acetate; CD16 PE: Cluster of differentiation 16 – Phycoerytherin; FITC: Fluorescein isothiocyanate.

7.8 Impact of organ-targeted therapies on neutrophil function

A reduction in serum ammonia was not achieved in patients on CVVH in either the CLD [Table 7-6] or the ALF cohorts and no significant difference was observed in neutrophil phagocytosis or oxidative burst parameters between the 2 cohorts. Patients on CVVH were generally more unwell as assessed by increasing APACHE II and SOFA scores even when adjusted for the presence of renal failure (both $P<0.001$) as well as increasing HE grade, vasopressor requirement and mechanical ventilation.

A single 51-year old patient with ALF secondary to acetaminophen overdose, requiring mechanical ventilation for high grade HE, underwent plasmapheresis. The patient was declined for LT due to psychiatric co-morbidity. Following 72-hours of plasmapheresis a reduction in arterial ammonia from 159 mmol/L to 66 mmol/L was achieved; with this *ex vivo* NPA improved from 69.3 to 94.0%. A reduction in levels of TNFα, IL-1β, IL-10, IL-17 were seen but not IL-6. The patient avoided further septic insult and survived to discharge. Plasmapheresis is therefore likely to be a more effective than conventional CVVH in reducing serum ammonia as well as removing other circulating humoral factors such as PAMPs, DAMPs and plasma cytokines.

Table 7-8 Effect of continuous veno-venous haemofiltration (CVVH) on plasma ammonia, neutrophil function and plasma cytokine concentrations in patients with cirrhosis complicated by acute-on-chronic liver failure.

	No CVVH (N=9)	**CVVH (N=8)**	**p-values**
Plasma ammonia concentration (μmol/L)	85 (73-101)	65 (46-83)	0.25
Neutrophil phagocytic activity (%)	64 (52-69)	68 (43-75)	0.75
Neutrophil spontaneous oxidative burst (%)	10 (5-38)	24 (6-55)	0.71
Neutrophil *E.coli* stimulated burst (%)	85 (81-92)	87 (74-96)	1.00
Neutrophil resting forward scatter geometric MFI	6.87×10^4 (6.51×10^4-7.24×10^4)	7.69×10^4 (7.46×10^4-7.34×10^4)	0.12
TNFα pg/mL median (IQR)	63 (48-225)	99 (44-178)	0.83
IL-1β pg/mL median (IQR)	7333 (1617-27841)	17241 (1773-73433)	0.81
IL-6 pg/mL median (IQR)	66 (44-168)	202 (73-2480)	0.21
IL-8 pg/mL median (IQR)	185 (153-616)	433 (197-618)	0.83
IL-10 pg/mL median (IQR)	155 (74-296)	766 (233-3268)	0.25
IL-17 pg/mL median (IQR)	244 (103-989)	597 (83-906)	1.00

7.9 Discussion

These data show that circulating neutrophils in patients with cirrhosis display an activated phenotype (increased surface expression of CD11b and loss of CD62L) with a reduced capacity to phagocytose microbes and impairment of chemotactic migration before and after fMLP stimulation. An elevated spontaneous production of ROS from circulating neutrophils is present in a third of cirrhotic patients and this deteriorates further following the development of HE and AoCLF suggesting a putative role in the development of organ failure. Furthermore, circulating neutrophil dysfunction in cirrhosis predicts infection and 90-day survival in stable cirrhosis.

An enhanced expression of CD11b, which in combination with CD18 forms the dimeric Mac-1, was found on the surface of circulating neutrophils in cirrhosis. In addition circulating neutrophils show a loss of surface CD62L (L-selectin), but unchanged levels of CD16, indicating a primed neutrophil phenotype. These findings mirror those of Rosenbloom et al who showed an increasing surface density and avidity of the complement receptors CD11b and CD35 on circulating neutrophils in cirrhosis correlating with increasing serum IL-6 levels. (Rosenbloom et al., 1995) CD11b levels correlated with the intensity of SOB and engagement of CD11b on neutrophils increased ROS production. (Nathan et al., 1989) Determination of an expanded range of neutrophil surface receptors would help further characterise neutrophil phenotype and confirm a sepsis-like phenotype but in addition might identify patients at increased risk of developing infections such as SBP and dynamic changes in neutrophil phenotype might serve as biomarkers to prognosticate. Such work should include CD62L expression in an expanded cohort of patients, β-integrins, chemokine receptors e.g. CXC2, CD49d, CD64 and TLRs.

The declining NPA observed in patients with cirrhosis was found to be associated with increasing severity of liver disease as measured by Child-Pugh class, supporting previous findings of other groups. (Rajkovic and Williams, 1986, Tritto et al., 2011) Nevertheless, phagocytic dysfunction to some degree appears to be a universal phenomenon observed in most patients with cirrhosis.

Other groups have reported neutrophil phagocytosis to be normal (Kirsch et al., 2000, Panasiuk et al., 2005) or even increased in cirrhosis. (Neugebauer et al., 2008) The apparent discrepancy in observations between studies may be explained by enhanced rates of neutrophil apoptosis due to poor handling, sample contamination with endotoxin

or the use of ammonia chloride for red cell lysis. In addition it is important to exclude cigarette smokers and those who have undertaken vigorous exercise in the last 24 hours from the healthy control cohort as these factors can impair NPA/NPC.

Overall we report a reduction in the percentage of neutrophils undergoing phagocytosis (phagocytic activity) but Tritto et al mirror our findings using the geometric median uptake of *E.coli* by neutrophils (E.coli GMFI [phagocytic capacity]) showing that either measure is valid.

Paradoxically, our data suggests active alcohol consumers have higher levels of NPA than abstinent drinkers with comparable severity of liver disease, which may be explained by enhanced neutrophil priming through increased bacterial translocation from the gut. (Bode et al., 1987).

The observed association between elevated plasma ammonia concentration and impaired NPA replicates Dr Shawcross and colleagues previous observations. (Shawcross et al., 2008a) Acute increases in plasma arterial ammonia concentration induced by a simulated upper gastrointestinal bleed have been shown to induce neutrophil swelling and phagocytic dysfunction in patients with stable cirrhosis. The adverse impact of ammonia exposure on neutrophil function can be abrogated *ex vivo* following incubation of the neutrophils with an agonist of the p38-MAPK pathway. (Shawcross et al., 2008a) It has been postulated that ammonia-induced neutrophil swelling induces phosphorylation of p38-MAPK which serves as an important cell osmoregulator. (vom Dahl et al., 2001) p38-MAPK is also upregulated during neutrophil priming and activation, with down-stream effects including the promotion of apoptosis and the activation of pro-inflammatory transcription factors.

Further support of a central-role for activated p38-MAPK in NPA comes from the observation that in patients with diuretic intolerant/resistant ascites the concomitant use of propranolol was associated with worsening of NPA. This may offer an explanation as to why Serste and colleagues recently identified increased mortality in patients with refractory ascites treated with propranolol. (Serste et al., 2010) Propranolol increases neutrophil motility by promoting increased intracellular cyclic GMP activity (Anderson and van Rensburg, 1979) and thus might be postulated to have a beneficial effect in cirrhosis. It can however increase p38-MAPK phosphorylation and therefore potentially further suppress neutrophil phagocytic function. (Busse and Sosman, 1984) Reduced NPA in concert with increased bacterial DNA translocation from the gut into the portal vein in patients with refractory ascites might thereby culminate in hepatic endothelial

dysfunction, systemic endotoxemia, and resultant systemic inflammatory response. (Bellot et al., 2010)

The finding however, that elevated serum transaminases and hyperbilirubinaemia are associated with higher plasma levels of IL-17 and a lower phagocytic capability in AoCLF supports hepatic inflammation as being an important driver of neutrophil phagocytic dysfunction. IL-17 is a critical mediator of neutrophil recruitment. IL-17 secretion from effector $CD4^+$ T-cells is thought to be an important mediator of innate immune function and the development of chronic inflammation (Qian et al., 2010) and IL-17 secreting hepatic cell infiltrates have been shown to be present in patients with acute alcoholic hepatitis. (Lemmers et al., 2009)

The finding of an enhanced spontaneous production of ROS from circulating neutrophils in patients who go on to develop infection and organ dysfunction or HE may hold the key to understanding why some patients with cirrhosis are susceptible to developing MODS and HE. Indeed, cirrhotic patients who have increased basal production of ROS appear to be at the highest risk for developing complications and MODS. This is likely to be driven by systemic pro-inflammatory milieu that bathes these neutrophils with elevated levels of TNFα, IL-6, IL-8 and IL-10 generated principally as a consequence of bacterial infection, with complicating infection being seen in 23% of patients with HE and 58% of patients with AoCLF, enhanced bacterial translocation of PAMPs across a leaky gut (Neugebauer et al., 2008) or from 'sterile' hepatic inflammation (DAMPs such as HMGB-1) may explain other cases. These findings support the hypothesis that bacterial driven (PAMPs) or 'sterile' hepatic inflammation induce a dysfunctional response in circulating neutrophils and through homing to other endothelial beds can induce oxidative stress and organ-injury. Similar findings have been shown in patients with acute alcoholic hepatitis where an increase in SOB is seen which correlates with severity of liver disease. (Parlesak et al., 2003, Mookerjee et al., 2007b) Interestingly the phenomenon of increased SOB appears to be independent of plasma ammonia levels. There is a suggestion that immunosuppressive therapies may reduce neutrophil SOB as patients with autoimmune liver disease, many of whom were immunosuppressed, had reduced SOB compared to other aetiologies of liver disease and the effect of corticosteroid therapy in patients with acute alcoholic hepatitis deserve further study.

Perhaps the most powerful observation of this study is that baseline circulating neutrophil dysfunction is a robust predictor of outcome and 90-day and 1-year survival.

Baseline resting neutrophil OB of ≥12% predicted 90-day survival with an AUROC of 0.81, with survivors at 90-days having a significantly lower resting OB. Impaired NPC is also an important determinant of 90-day mortality with an AUROC of 0.83 with survivors at 90-days and 1-year having a significantly higher NPC. This implies that neutrophil biomarkers can predict medium-term (90-day) and longer-term (1-year) outcome in patients with stable cirrhosis. This also supports the findings of Mookerjee and colleagues who showed neutrophil dysfunction to be an important outcome predictor in acute alcoholic hepatitis. (Mookerjee et al., 2007a)

Overall the number of samples analysed for chemotaxis was small so it was not possible to draw firm statistically significant conclusions. Intriguingly, a heterogeneous response was seen with a number of patients with cirrhosis, either stable or complicated by HE or AoCLF, showing either baseline neutrophil activation or increased migration with a paradoxical defective migration ability following exposure to the chemokine fMLP. No apparent association with plasma arterial ammonia concentration was seen. This supports earlier findings by Fiuza et al who showed an impaired ability of neutrophils in patients with cirrhosis to migrate using a skin window technique. (Fiuza et al., 2000)

It is interesting to note that neutrophil phagocytosis shows ongoing derangement 72-hours post-LT associated with persisting elevations in levels of pro- and anti-inflammatory cytokines. The ongoing neutrophil dysfunction may be multi-factorial including persisting neutrophil maladaptation due to cytokines, wound repair, hepatic ischaemia-reperfusion injury and immunosuppression. The persistence of phagocytic dysfunction in the early post-transplant phase may explain the ongoing predisposition to infection.

It is surprising that haemodialysis does not appear to have an effect on neutrophil phagocytic dysfunction; however, our study was underpowered to detect this change. It has been shown that CVVH can reduce plasma ammonia levels with greater ammonia clearance achieved with higher filtration volumes. (Slack et al., 2014) So it may be too early to dismiss this approach which merits further investigation. Other therapies such as albumin dialysis may in addition modulate neutrophil function and mandate investigation.

The evaluation of therapies that directly or indirectly target neutrophil dysfunction in patients with cirrhosis may therefore have a profound impact on reducing the inevitable development of infection, organ dysfunction and mortality in this population.

Chapter 8 - The role of the p38-mitogen activated protein kinase pathway in mediating circulating neutrophil dysfunction in liver failure

8.1 Introduction

Serum ammonia levels are raised in ALF and liver cirrhosis due to a reduced urea synthesis capacity and porto-caval shunting. It has recently been shown by Shawcross et al. that ammonia at physiological concentrations commonly seen in acute and chronic liver failure causes neutrophil swelling, leading to a reduced phagocytic capacity and high spontaneous OB (Shawcross et al., 2008b). These results were replicated in patients with cirrhosis with induced hyperammonemia suggesting a direct toxic effect of ammonia on neutrophils; an effect exacerbated by the presence of hyponatraemia.

Ammonia has also been shown to depress neutrophil chemotaxis (Coppi and Niederman, 1989) and can impair neutrophil energy metabolism, through inhibition of phosphate-dependent glutaminase (Sbarra and Karnovsky, 1959)

The susceptibility of neutrophils to ammonia is used to the advantage of some microorganisms and has been shown to be a virulence factor in *helicobacter pylori* infection and human periodontal infection. (Mayo et al., 1997, Niederman et al., 1990)

The p38-MAPK intracellular signalling pathway appears to be important in mediating this ammonia-induced dysfunction. Phosphorylation of p38-MAPK is a critical step in the neutrophil response to stress: it functions as a key osmoregulator, regulator of inflammatory gene expression (e.g. TNFα, IL-1 and IL-12), promotes phagocytosis and can lead to activation of neutrophil apoptotic pathways. (Zu et al., 1998, vom Dahl et al., 2001, Zarubin and Han, 2005) Activation of p38-MAPK signalling occurs through a kinase cascade following surface receptor ligation. Neutrophil p38-MAPK appears to be specifically activated by TNFα, GM-CSF, fMLP, PMA and inomycin. (Doyle et al., 2004, Zu et al., 1998, Hidari et al., 1997) SB203580 is a specific inhibitor of p38-MAPK activation and reduces the production of IL-8 and superoxide following neutrophil stimulation by TNFα (Zu et al., 1998) in addition to reducing neutrophil chemotaxis in response to fMLP.

The hypothesis that ammonia impairs neutrophil function by inducing cell-swelling resulting in elevated levels of phosphorylated p38-MAPK (the activated form) leading to activation of homeostatic mechanisms to correct cell swelling and induction of transcription of inflammatory genes within neutrophils [Figure 4-1] was tested indirectly by correlating blood ammonia concentration with neutrophil swelling assessed through flow-cytometry forward scatter, using a method previously reported for hepatocyte cell volume regulation, (Carini et al., 1999) and directly by measuring the neutrophil cross-

sectional area utilising transmission electron-microscopy (TEM). The effects of p38-MAPK modulators on neutrophil function and evaluation of activated and total p38-MAPK levels were also performed.

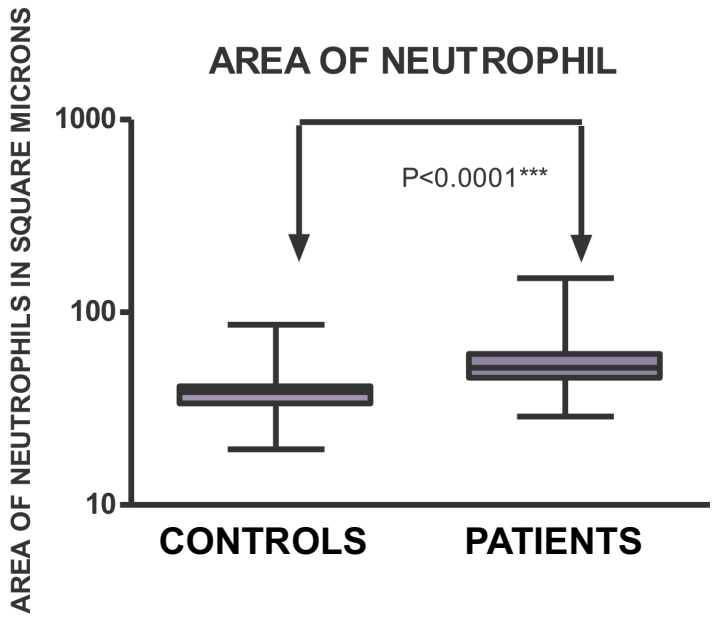
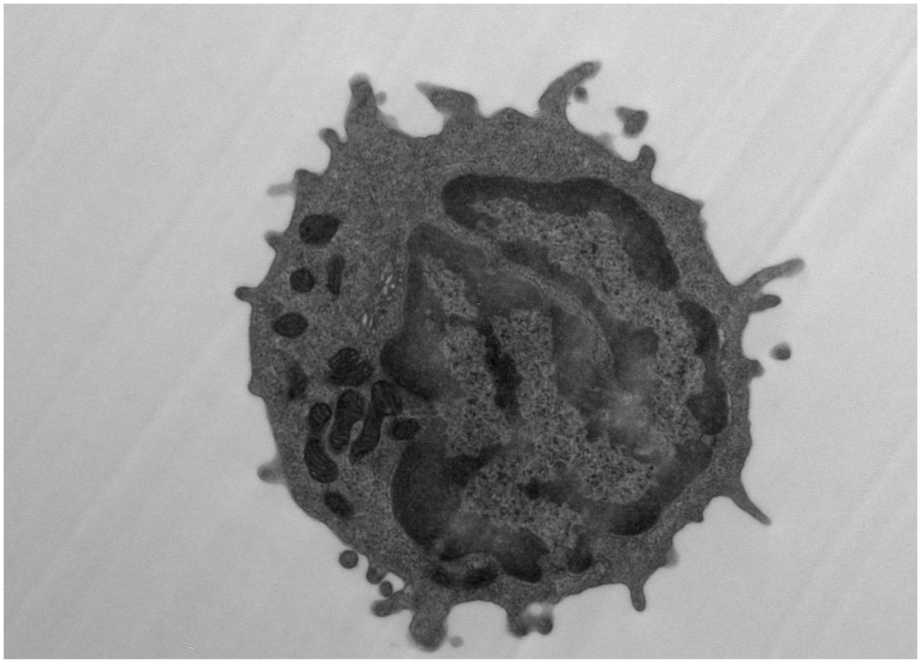


FIGURE 8-1 NEUTROPHIL CROSS-SECTIONAL AREA DETERMINED BY TRANSMISSION ELECTRON MICROSCOPY ON ISOLATED NEUTROPHILS FROM PATIENTS WITH CIRRHOSIS (N=3) AND HEALTHY CONTROLS (N=2).

Differences were compared using the Mann-Whitney U test ($p<0.0001$)

A



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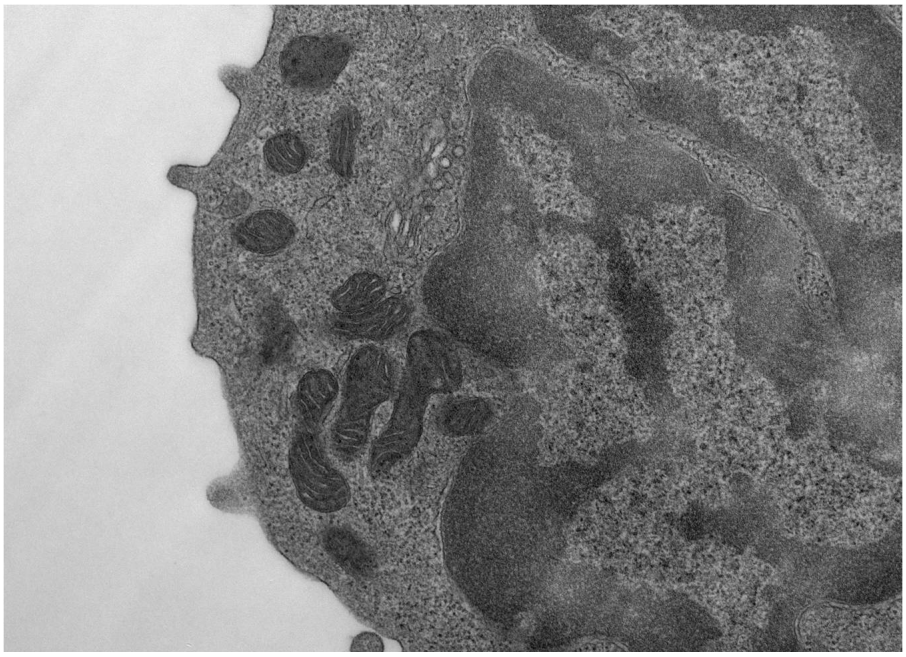
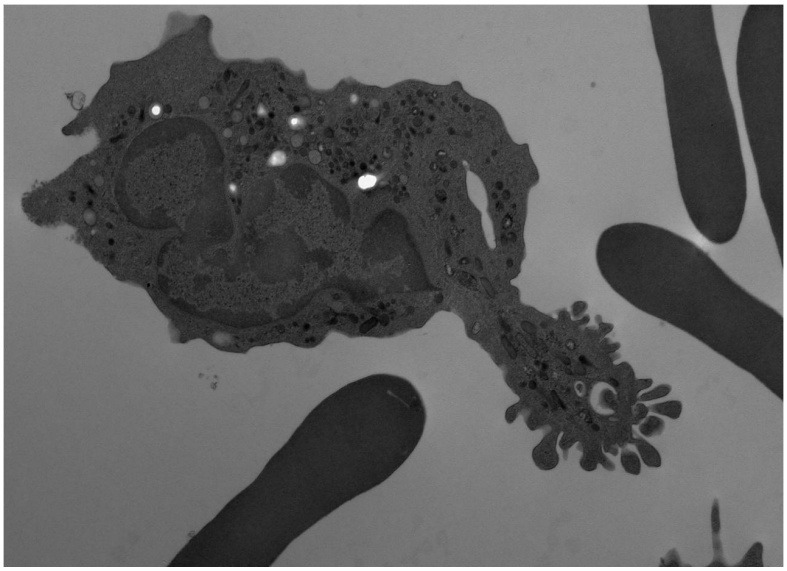


FIGURE 8-2 TRANSMISSION ELECTRON MICROGRAPH OF A RESTING NEUTROPHIL ISOLATED FROM A HEALTHY CONTROL, (A - 4800X AND B - 9300X)

These healthy neutrophils have a spherical shape, multi-lobed nucleus, dense cytoplasm and multiple mitochondria.

A



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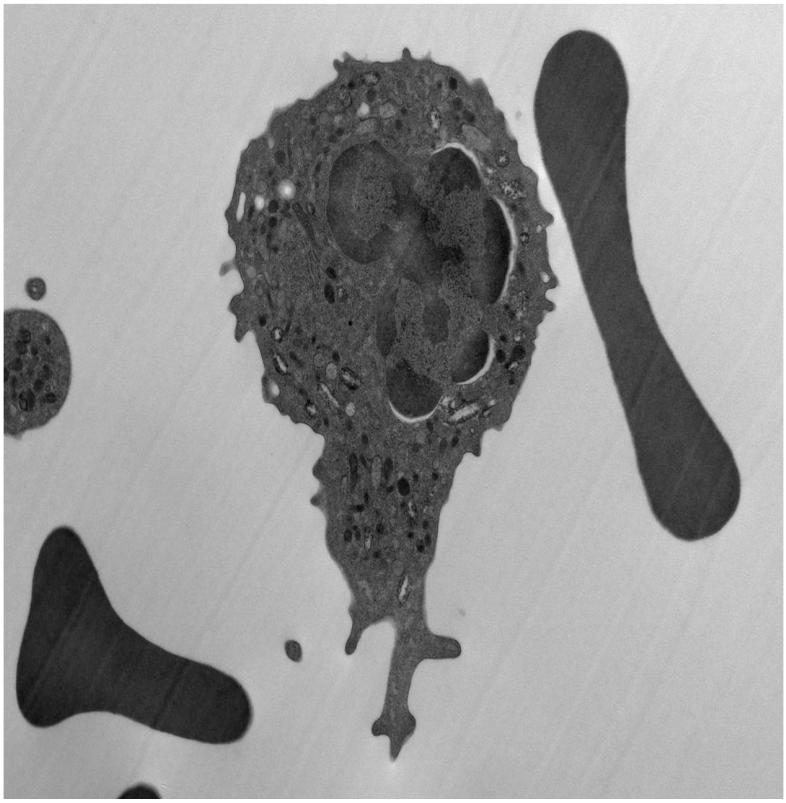

FIGURE 8-3 TRANSMISSION ELECTRON MICROGRAPHS OF A NEUTROPHIL PSEUDOPODIUM ENVELOPING OPSONISED *E.COLI* ISOLATED FROM A HEALTHY CONTROL WITH SURROUNDING ERYTHROCYTES (2900X)

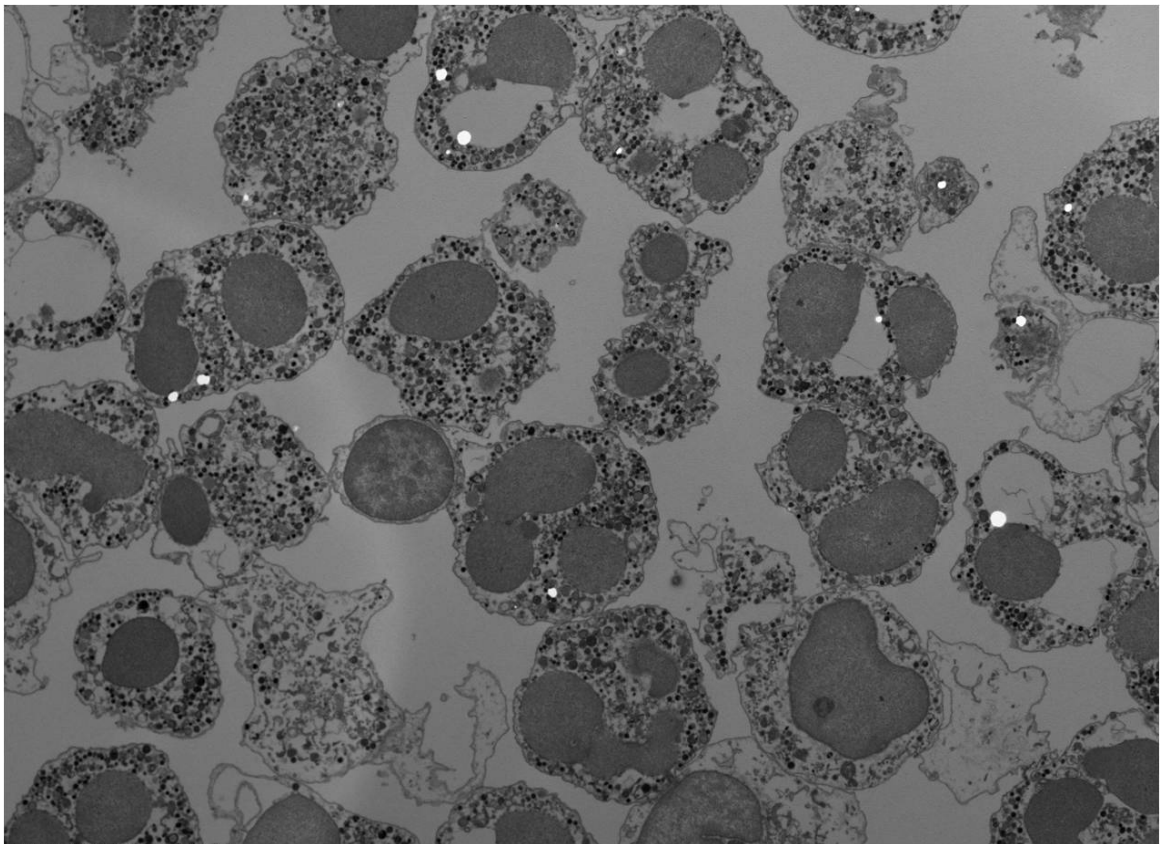

FIGURE 8-4 TRANSMISSION ELECTRON MICROGRAPH OF RESTING NEUTROPHILS ISOLATED FROM A PATIENT WITH ACUTE ALCOHOLIC HEPATITIS (890X)

This micrograph shows a loss of spherical shape with a highly granular cytoplasm and large pale nuclei suggesting reduction in nuclear chromatin. A single large vacuole is present.

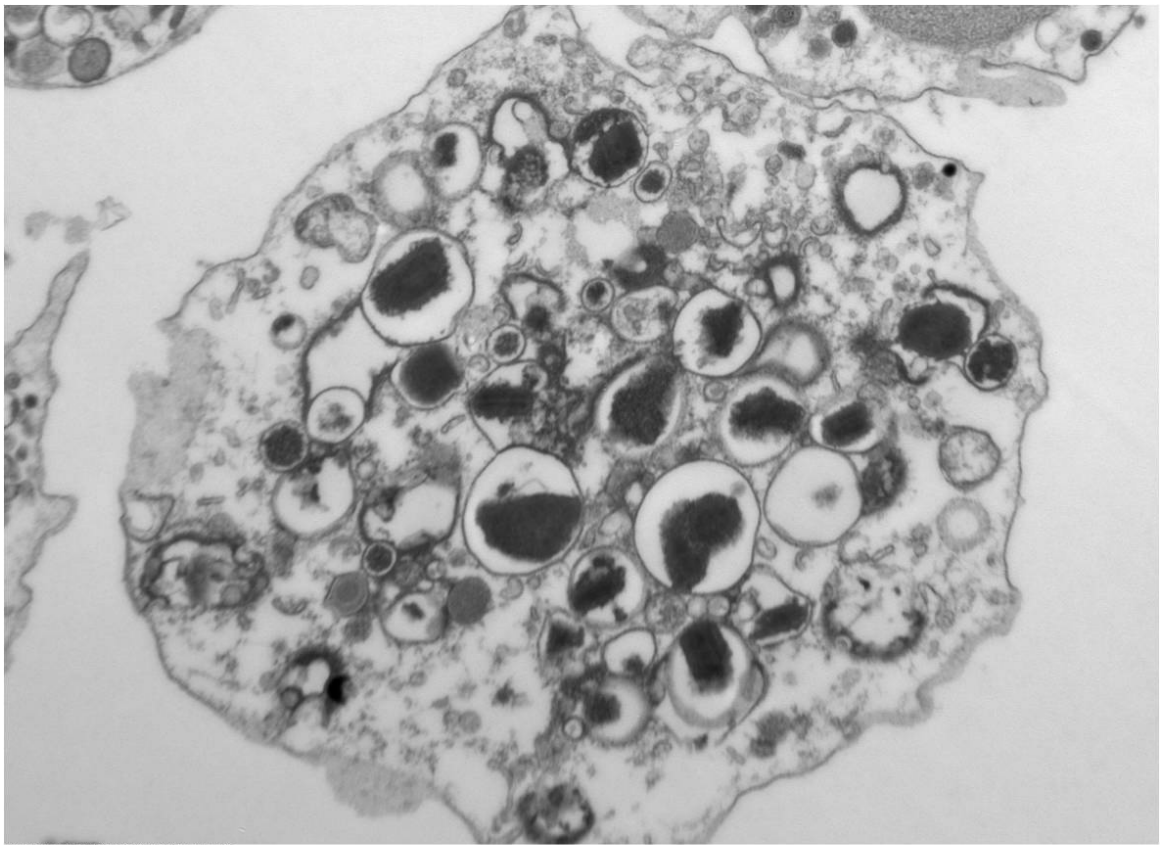

FIGURE 8.5 TRANSMISSION ELECTRON MICROGRAPH OF A NEUTROPHIL ISOLATED FROM A PATIENT WITH SUB-ACUTE LIVER FAILURE (4800X)

This micrograph shows extensive vacuolisation, pale cytoplasm and reduction in cytoplasmic granules in a patient with sub-acute liver failure secondary to hydralazine.

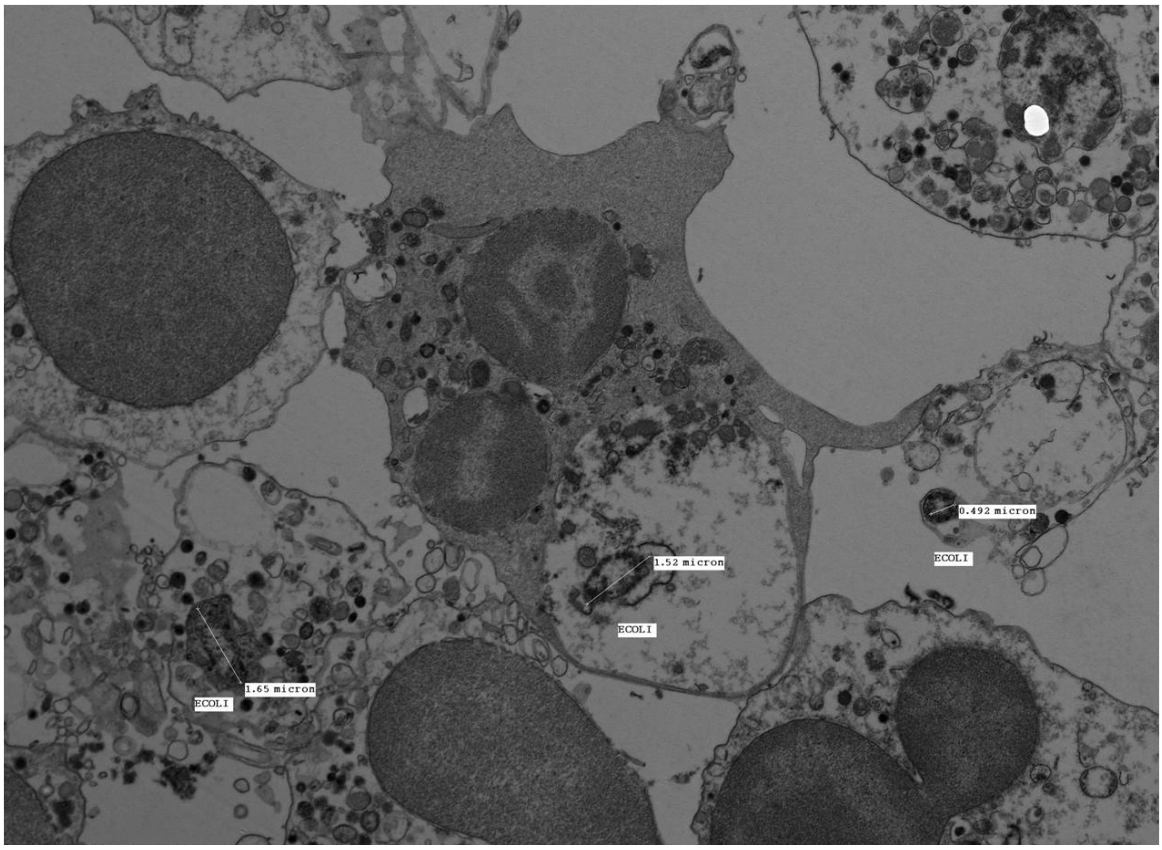

FIGURE 8-6 TRANSMISSION ELECTRON MICROGRAPH OF A NEUTROPHIL ISOLATED FROM A PATIENT WITH CHILD-PUGH B ALCOHOL-RELATED CIRRHOSIS (ABSTINENT) FOLLOWING PHAGOCYTOSIS OF OPSONISED *E.COLI.* (1900X)

This micrograph shows a neutrophil with poorly defined shape, pale cytoplasm and sparse mitochondria.

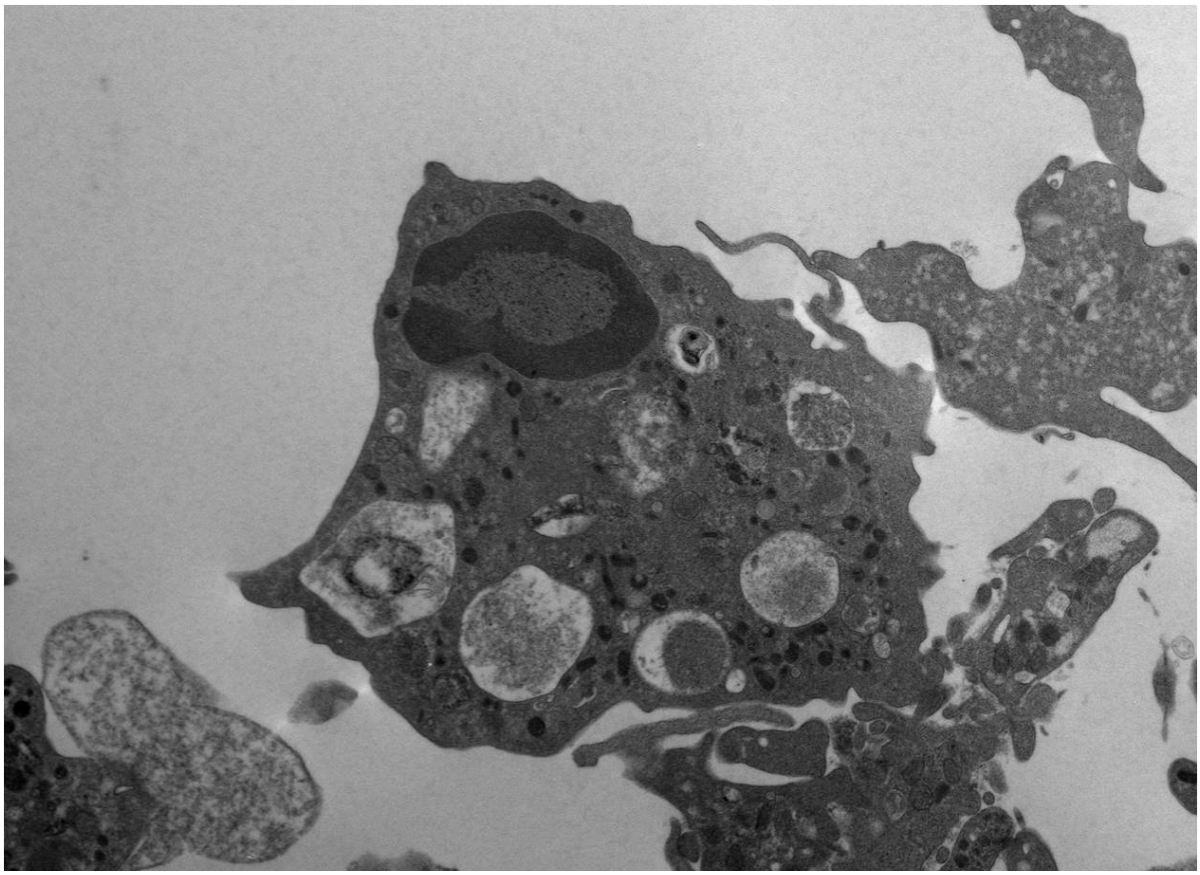

FIGURE 8-7 TRANSMISSION ELECTRON MICROGRAPH OF A NEUTROPHIL ISOLATED FROM A PATIENT WITH CHILD-PUGH B ALCOHOL-RELATED CIRRHOSIS (ACTIVE DRINKER) FOLLOWING EXPOSURE TO OPSONISED *E.COLI.* (2900X)

This micrograph shows neutrophil phagosomes surrounded by granules (toxic granulation) with loss of the normal rounded neutrophil shape.

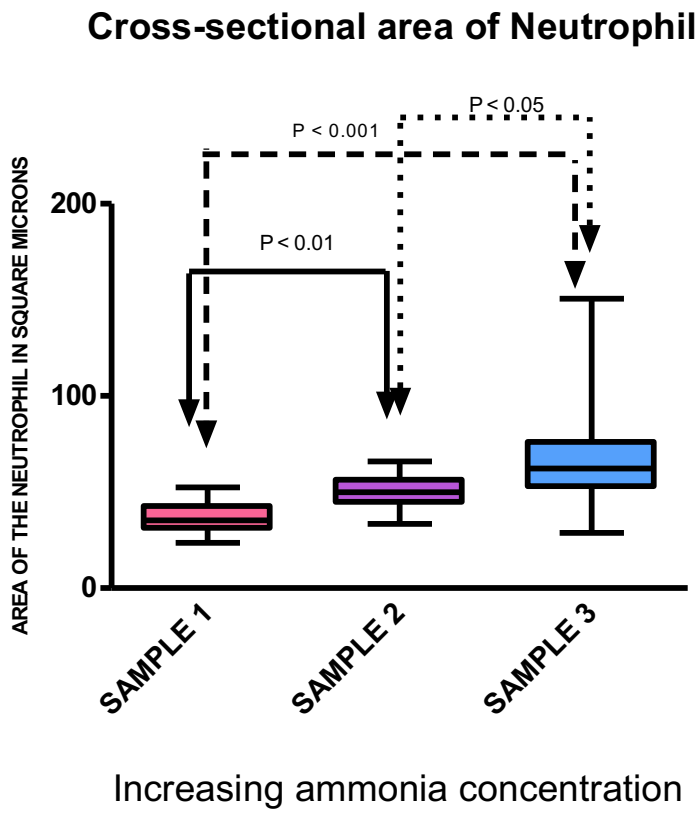

FIGURE 8-8 GRAPH SHOWING AVERAGE NEUTROPHIL SURFACE AREA COMPARED TO PLASMA AMMONIA IN A HEALTHY CONTROL AND 2 PATIENTS WITH CIRRHOSIS.

Measurements of 12 representative neutrophils were obtained for each sample with bars showing the mean and standard error of the readings.

Sample 1 - healthy control (plasma ammonia concentration 13μmol/L); Sample 2 - patient with cirrhosis and low ammonia concentration (35μmol/L); Sample 3 - patient with cirrhosis and high ammonia concentration (135μmol/L). Differences between the healthy control and patients were calculated using the Kruskal-Wallis test with Dunn's multiple comparison test.

8.2 Study to determine whether ammonia contributes to neutrophil swelling and dysfunction through changes in the p38-MAPK pathway

Ten ALF patients, with a median SOFA score of 16 (IQR 14-17) and APACHE II score of 22 (IQR 19-25), and ten patients with cirrhosis, with a median MELD score 27 (IQR 16-38) had blood drawn and incubated with neutrophil modulators. The median arterial ammonia level in the ALF group was 88 μmol (IQR 44-158; 9/10 on haemofiltration) and cirrhosis group was 62 μmol (49-99) μmol/L. Baseline NPA was 67% (IQR 54-75) in the ALF group and 72% (39-84) in the CLF group compared to >85% in healthy controls.

Ex vivo incubation of isolated neutrophils with 200μmol/L ammonium chloride for 90-minutes taken from these patients reduced NPA by 7.3 % (IQR 3.3-14.3, $p<0.05$) [Figure 8-8]. The p38-MAPK agonist isoproterenol [Sigma, UK] (2 μmol/L) significantly abrogated the ammonia-induced phagocytic impairment and improved bactericidal capacity ($p<0.007$). The specific p38-MAPK antagonist SB203580 [Calbiochem, UK] (40 μmol/L) however, exacerbated the ammonia-induced reduction in neutrophil phagocytic activity by a median of 5.2% (IQR 1.4-15.6); $p=0.003$ [Figure 8-8]. Flow cytometer plots showing the changes in neutrophil characteristics following exposure to p38-MAPK modulators are shown in Figure 8-9. There appears to be a robust dose response relationship between the SB203580 induced suppression of NPA and the enhancement of NPA seen with isoproterenol [Figure 8-10].

Ammonia was shown to supress both PMA and *E.coli* stimulated SOB but interestingly appeared to be a more potent inhibitor of PMA-induced burst than *E. coli* SOB [Figure 8-11]. PMA is known to activate p38-MAPK following activation of PKC and subsequent phosphorylation and ammonia's effects therefore appears to be specific to this pathway whereas *E. coli* can still generate a burst via alternative pathways. Isoproterenol (2 μmol/L) reversed the ammonia-induced increase in neutrophil SOB ($p<0.001$) and at the same time reduced the ability of neutrophils to generate an ESOB ($p<0.05$) [Figure 8-11 and 8-12]. SB203580 (40 μmol/L) did not appear to further exacerbate the ammonia-induced impairment in neutrophil SOB and ESOB.

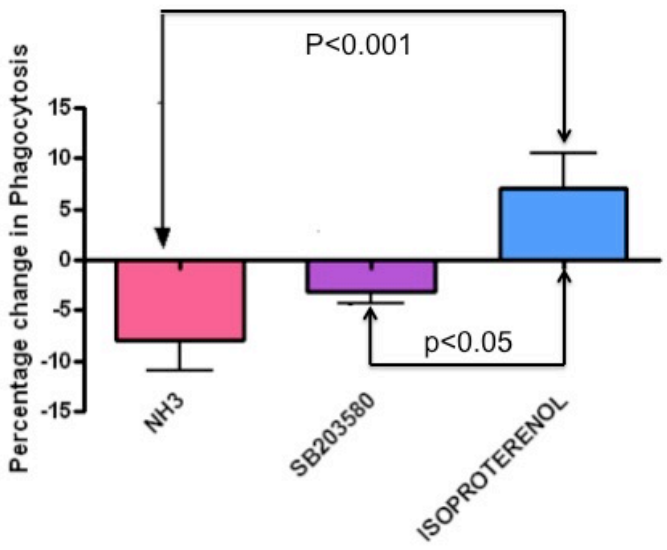

FIGURE 8-9 THE EFFECT OF A 90-MINUTE PRE-INCUBATION WITH AMMONIA WITH AND WITHOUT THE P38-MAPK MODULATORS SB203580 AND ISOPROTORENOL ON NEUTROPHIL PHAGOCYTIC ACTIVITY IN 10 PATIENTS WITH CIRRHOSIS AND 10 PATIENTS WITH ALF

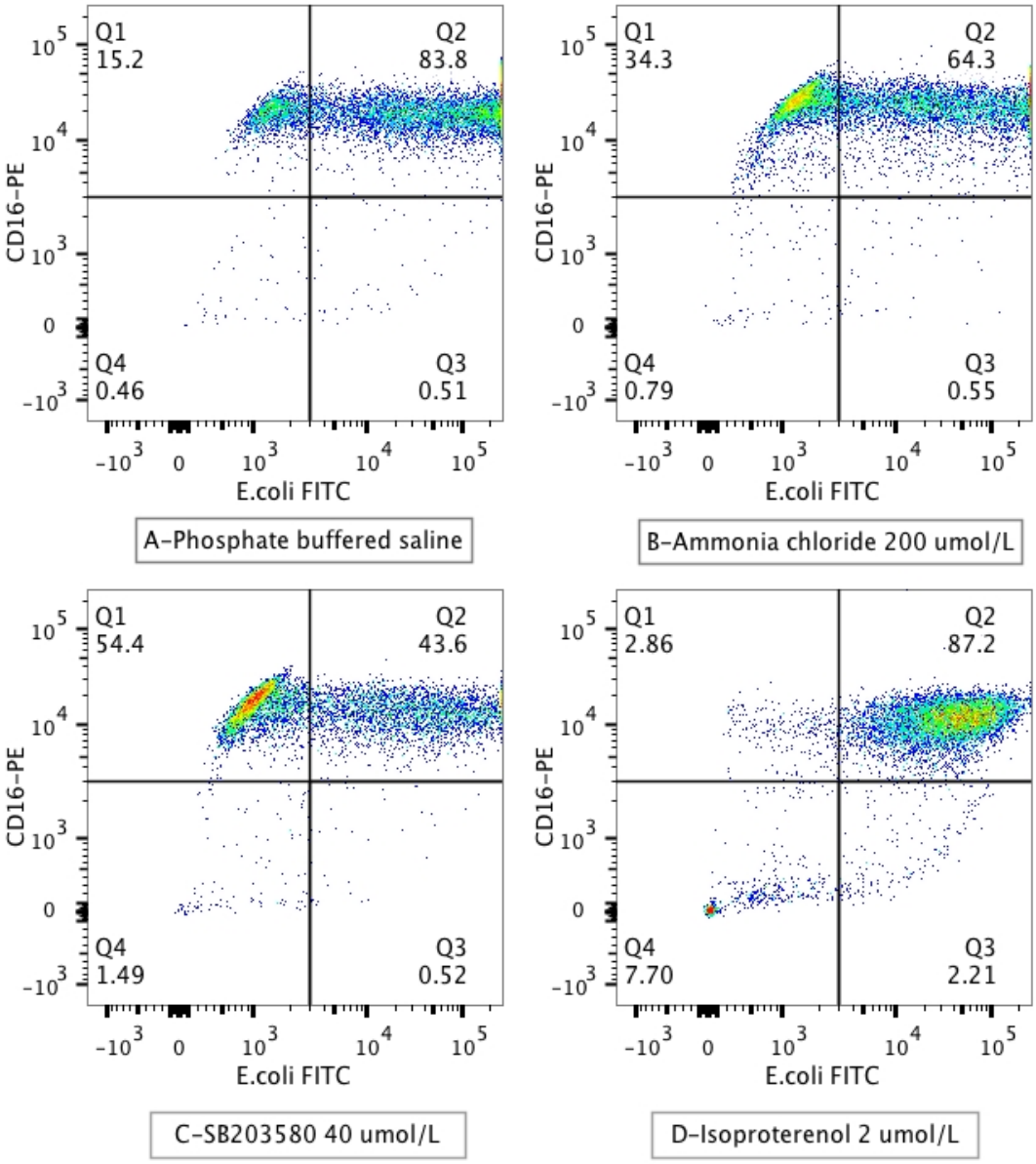

FIGURE 8-10 FLOW PLOTS OF NEUTROPHIL PHAGOCYTOSIS FOLLOWING 90-MINUTE INCUBATION WITH P38-MAPK MODULATORS.

Granulocytes were gated on forward and side scatter characteristics and neutrophils identified by labelling with CD16-PE. Neutrophil phagocytosis was determined by uptake of opsonised *E.coli* labelled with FITC. Prior to neutrophil function testing neutrophils were pre-incubated for 90-minute with: A- phosphate buffered saline (control); B- Ammonium chloride 200 µmol/L; C- SB203580 40 µmol/L (Calbiochem, UK); D-Isoproterenol 2 µmol/L (Sigma, UK).

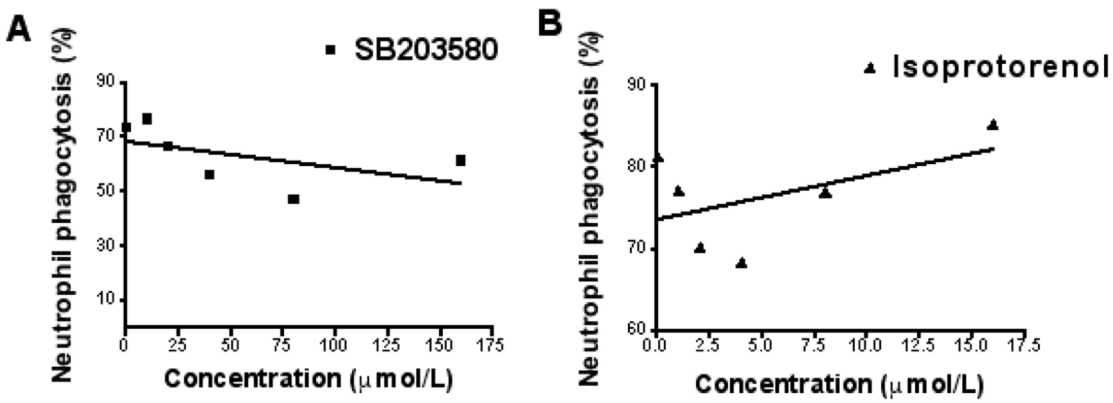

FIGURE 8-11 EFFECT OF INCREASING CONCENTRATIONS OF THE P38-MAPK MODULATORS ISOPROTERENOL (A) AND SB203580 (B) ON NEUTROPHIL PHAGOCYTOSIS.

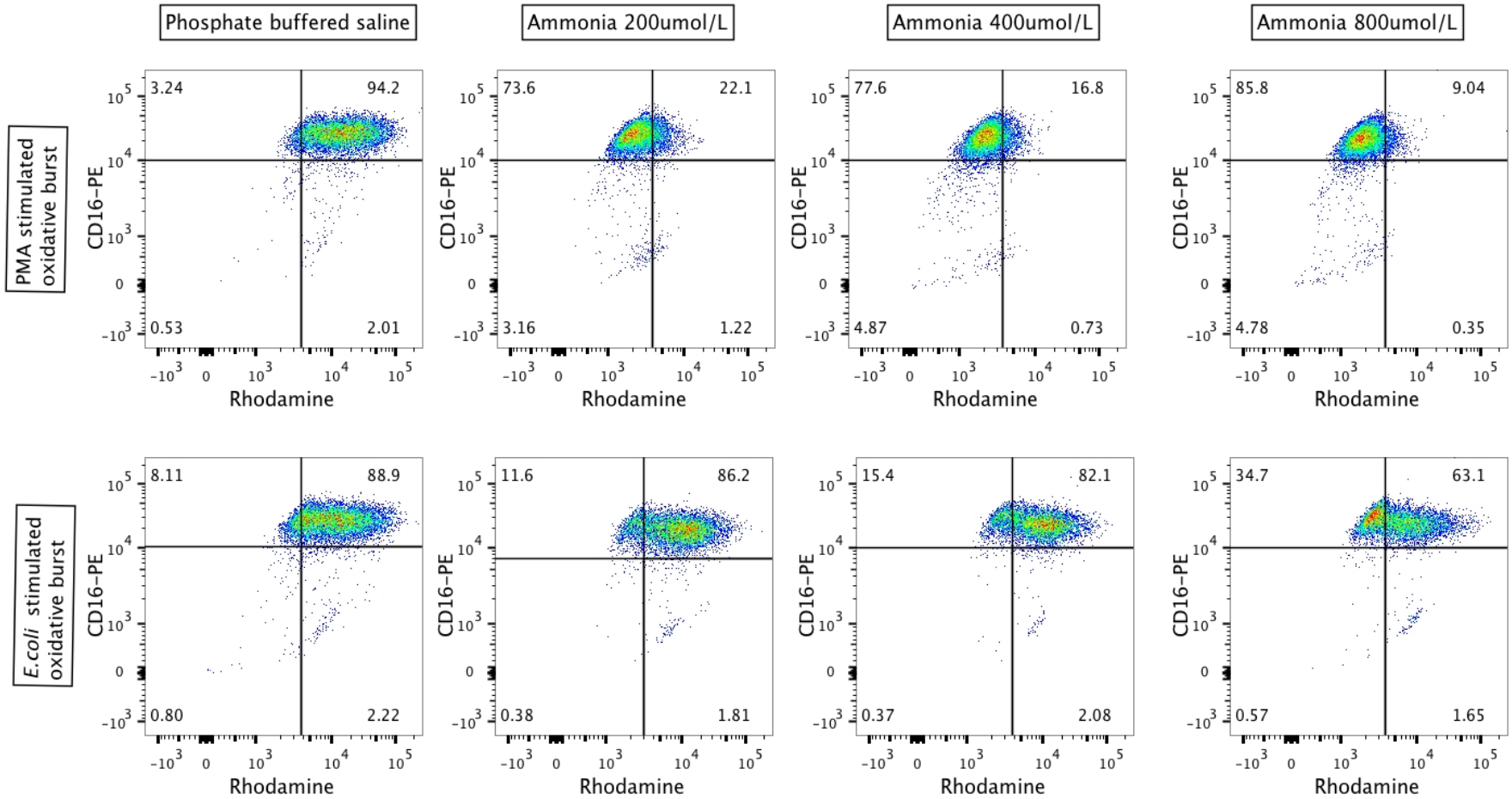

FIGURE 8-12 FLOW-PLOTS OF NEUTROPHIL PHORBOL MYRISTATE ACETATE AND *E.COLI* STIMULATED OXIDATIVE BURST FOLLOWING 90-MINUTE INCUBATION WITH PHOSPHATE BUFFERED SALINE AND INCREASING CONCENTRATIONS OF AMMONIUM CHLORIDE.

These plots show that there is an early defect in PMA-induced oxidative burst at ammonium chloride concentrations of 200 μmol/L but *E.coli* stimulated oxidative burst shows a progressive decline as ammonium chloride concentration increases. This suggests *E. coli* activates neutrophil oxidative burst through alternative pathways to PMA and is only impaired at toxic levels of ammonium chloride.

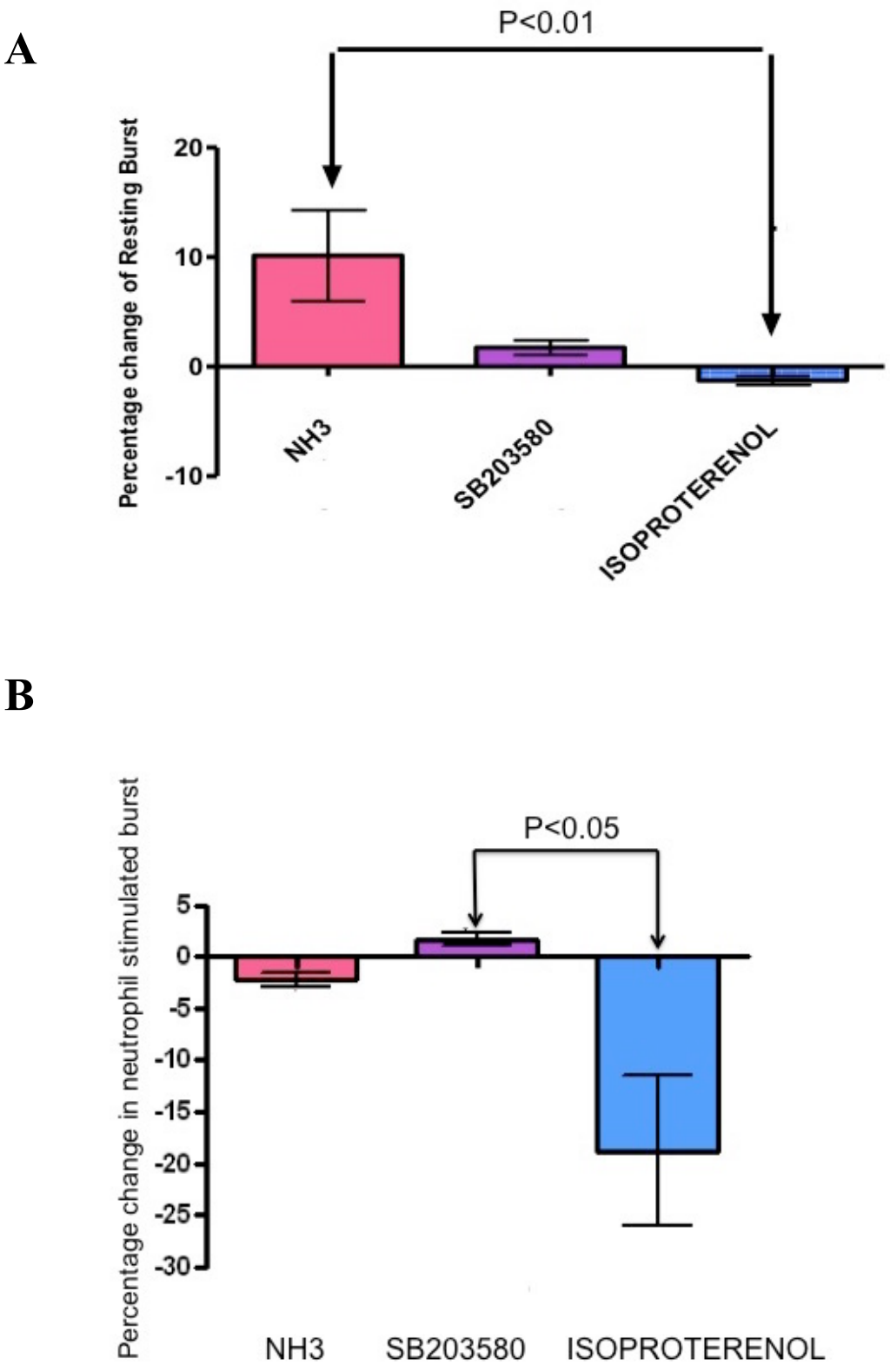

FIGURE 8-13 THE EFFECT OF A 90-MINUTE PRE-INCUBATION WITH AMMONIA AND THE P38-MAPK MODULATORS SB203580 AND ISOPROTERENOL ON NEUTROPHIL RESTING (A) AND *E.COLI* STIMULATED (B) OXIDATIVE BURST IN 10 PATIENTS WITH CIRRHOSIS AND 10 PATIENTS WITH ALF

8.3 Study to investigate activation of the p38-MAPK pathway in neutrophils isolated from patients with liver failure and the impact of p38-MAPK modulation.

Cytokine bead array assays of neutrophils were performed after a 90-minute incubation with PBS or 200μmol/L ammonium chloride and either PBS or one of the p38-MAPK modulators isoproterenol (2 μmol/L) and SB203580 (40 μmol/L). Samples had been stored at -80°C prior to the cytokine bead array assay being performed. Three healthy control samples, 5 patients with cirrhosis, 4 patients with ALF and one patient post-LT for ALF due to paracetamol were analysed. Samples were analysed on 2 separate occasions on the first there was a problem with the set-up of data acquisition on the flow cytometer as the PMT voltages were not set correctly and so the gates could not be correctly set for the plex analysis. On the second occasion excellent standard curves of the housekeeper protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphorylated and total p38-MAPK were achieved [Figure 8-15]. However, when analysis of samples was performed the housekeeper protein GAPDH was undetectable and the yield of both total and phosphorylated p38 was very low suggesting that the samples had deteriorated following storage [Table 8-1]. Unfortunately there was insufficient stored sample for further analysis and therefore no meaningful analysis of levels of p38-MAPK could be performed.

Table 8-1 Cytokine bead array of neutrophils from healthy controls and patients with liver disease after 90-minute incubation with p38-MAPK modulators.

Sample group	Control Phos p38-MAPK	Control total:Phos p38-MAPK	NH_3 Phos p38-MAPK	NH_3 total:Phos p38-MAPK	Iso Phos p38-MAPK	Iso total;Phos p38-MAPK	SB Phos p38-MAPK	SB total:Phos p38-MAPK
HC	11.5	2.1	5.7	2.0	4.4	3.4	3.5	2.0
Cirrhosis	12.8	3.6	14.1	3.2	5.0	3.1	4.6	3.4
ALF	17.1	3.9	11.8	3.0	4.9	2.9	4.3	3.2
Post-LT	12.3	3.5	10.4	2.5	3.7	3.3	3.9	1.7

A low yield of total and phosphorylated p38-MAPK (U/mL) was obtained likely representing background activity of the antibodies used in the experiment.

Abbreviations: HC: Healthy control; ALF: Acute liver failure; LT: Liver transplantation; Phos p38-MAPK: Phosphorylated p38-MAPK; Iso: Isoproterenol; SB: SB203580.

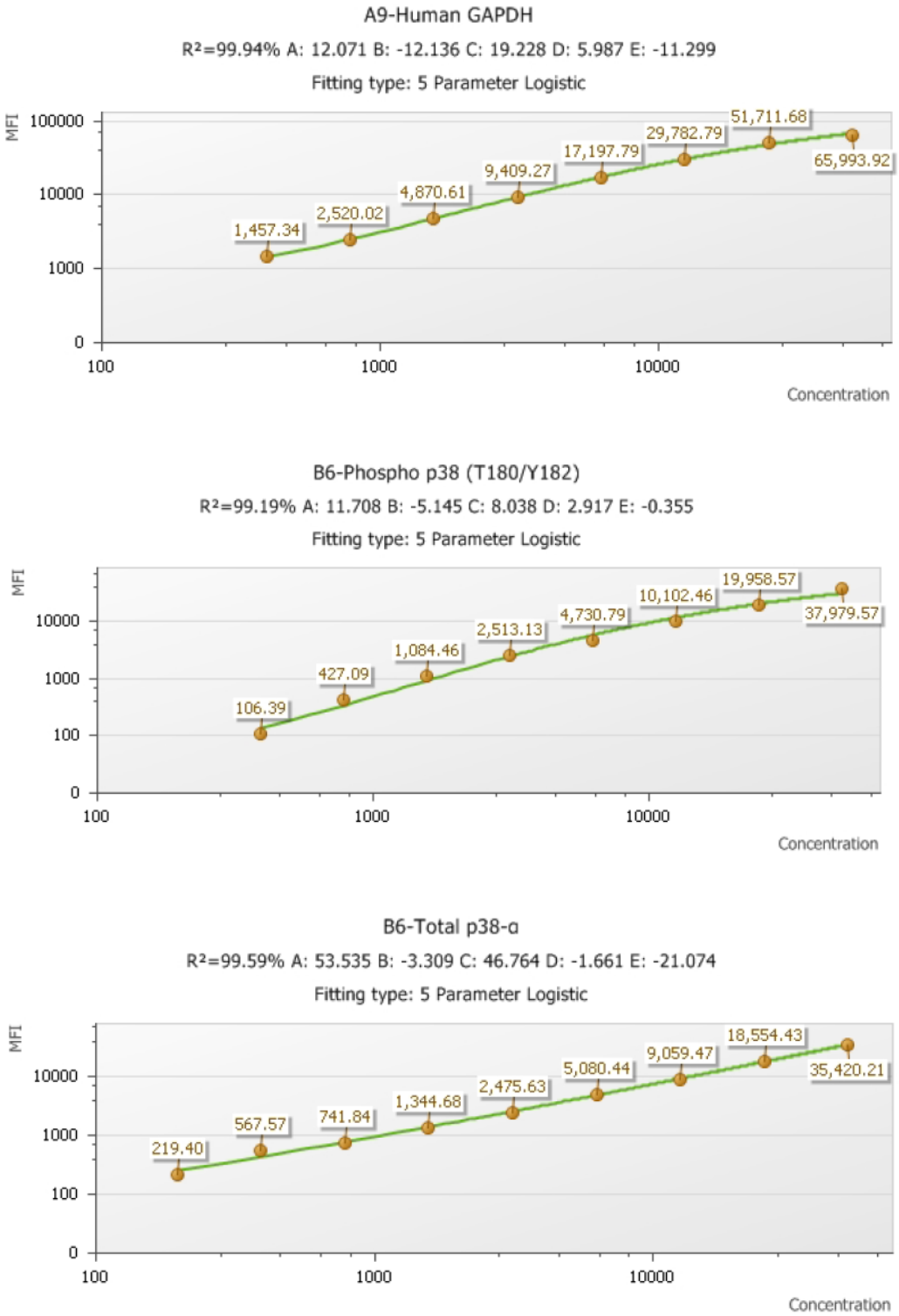

FIGURE 8-14 STANDARD CURVES OBTAINED FOR GADPH, TOTAL AND PHOSPHORYLATED P38-MAPK USING CYTOKINE BEAD ARRAY

(Standard curves plotted using 5-parameter logistic regression)

8.5 Discussion

These data show compelling evidence that neutrophils in patients with liver disease develop a 'swollen' phenotype with increased cell volume correlating with plasma ammonia levels. Furthermore, transmission electron microscopy studies have revealed profound abnormalities in the neutrophil ultrastructure with loss of rounded shape, extensive vacuolisation, pale cytoplasm and paucity of mitochondria. These findings in the setting of liver disease are novel and reveal that the neutrophils exist in a 'stressed' state probably as a consequence of DNA damage in response to elevated levels of ROS. This appearance is likely to represent neutrophil aging or 'senescence' whereby neutrophils undergo a change in the 'cell state' rendering them unable to respond to chemoattractants such as fMLP, undertake respiratory burst or to degranulate. (Whyte et al., 1993) Neutrophils are terminally differentiated short lived cells and cellular functions can be limited the mechanism of cellular senescence associated with up-regulation of CXCR4 on the neutrophil surface resulting in bone marrow homing and apoptosis. Neutrophil senescence occurs in parallel with spontaneous apoptosis, and indeed neutrophil apoptosis is thought to be the major factor in the functional senescence of neutrophils. (Lee et al., 1993) Neutrophil senescence in the setting of liver disease is likely to be secondary to ammonia-induced mitochondrial damage or ROS-associated with reduced apoptosis secondary to LPS stimulation, the secretion of GM-CSF secretion and pro-inflammatory cytokines. Neutrophil apoptosis is a critical event in the resolution of sepsis and is reduced in severe sepsis due to the presence of LPS, pro-inflammatory cytokines, colony stimulating factors and IL-10 possibly regulated by intracellular kinase-pathways. (Keel et al., 1997) In liver disease there is ongoing hepatic inflammation associated with chronic endotoxemia potentially setting up a 'perfect storm' to prolonge the life span of dysfunctional 'stressed' neutrophils. Interestingly a group that looked into a small populations of mainly HCV-related cirrhosis showed enhanced apoptosis in *ex-vivo* neutrophils (Ramirez et al., 2004) from decompensated cirrhotics compared to compensated liver disease. However, neutrophils need careful handling *ex-vivo* and sudden removal from a supportive environment where apoptosis is inhibited to an *ex-vivo* environment lacking support could trigger apoptosis so further studies in this area are required.

The most pivotal finding of the study is further tantalising evidence that activation of the p38 MAPK signal transduction system is an important protective mechanism for neutrophils and appears to be downregulated in acute and chronic liver disease. In addition healthy control neutrophils display a profound reduction in both PMA and *E coli* stimulated OB after exposure to increasing concentrations of ammonia; an effect abrogated by the p38 MAPK agonist isoproterenol. Kinase systems such as NF-κB and p38-MAPK are known to be critical in cellular immune activation and in the response to infection but their widespread distribution within mammalian cells limits their use as therapeutic targets. This study provides clear evidence that targeting the cytoprotective p38-MAPK pathway is a valuable strategy in cirrhosis with agonists showing the ability to reverse the ammonia-induced impairment to neutrophil phagocytosis while supressing neutrophils ability to burst. It is interesting to note that PMA induced burst was more specifically affected by ammonia than *E. coli* SOB. This give further support for the direct perturbation of this pathway by ammonia as PMA is known to activate p38-MAPK following activation of PKC and subsequent phosphorylation. Therefore p38-MAPK activation would allow antibacterial properties of the neutrophil to be maintained whilst avoiding ROS mediated bystander damage.

This is very exciting as p38-MAPK agonists potentially offer a targeted therapeutic strategy that can reverse the fundamental neutrophil defects in cirrhosis and potentially reduce rates of infection. Unfortunately due to failure of the cytokine bead array assay direct confirmatory evidence that ammonia inhibits neutrophils directly through this pathway could not be proven. The low level of proteins for analysis means that either there were problems in the protein extraction step or the final concentration of protein in the samples. Another factor that may have led to deterioration is that the denatured samples were stored for some months prior to analysis due to difficulty obtaining further CBA kits. In future I would perform the denaturation step, on appropriately exposed neutrophils, followed by protein estimation using the Bradford assay to ensure a consistent yield of denatured protein on fresh samples. Only once good standard curves had been achieved and flow-cytometer set-up optimised would I proceed with the CBA. In addition I would design the CBA plex to include other kinase systems e.g. ERK and JNK. A second confirmatory method such as western blotting could have been performed too act as a validation set.

Despite this difficulty, Shawcross et al. have previously shown up-regulation in phosphorylation of the p38-MAPK using western blotting in the absence of up-regulation of other kinase pathways following ammonia exposure. (Shawcross et al., 2008a) It would however, be important to confirm if this is a direct p38-MAPK effect secondary to cell volume changes or direct ammonia-induced toxicity and in particular to exclude the involvement of other kinase pathways. It remains unclear if ammonia causes neutrophil swelling through osmotic stress, or through direct effects on aquaporin molecules as seen in brain astrocytes. (Rama Rao and Norenberg, 2007). It is interesting to note that blocking antibodies directed towards aquaporin-9 in neutrophils inhibits pseudopodium formation and motility-related shape changes. (Loitto et al., 2002) Another possible mechanism whereby ammonia causes neutrophil dysfunction is through energy depletion within the neutrophil as they are reliant on the conversion of glutamine to glutamate a process inhibited by ammonia. (Sbarra and Karnovsky, 1959)

The previous effects of hyponatraemia seen in normal *ex vivo* neutrophils appears to be immaterial for neutrophils from patients with liver disease, failing to show significance on univariate analysis. The reasons for this are unclear but indicate a degree of neutrophil adaptation increasing resistance to the cell volume increases induced by hyponatraemia. It is interesting to note that hypernatraemia appears to modulate neutrophil function and increasing TLR expression in a murine model of thermal injury following the use of hypertonic saline. (Chen et al., 2006) Active correction of hyponatraemia and mild hypothermia is a standard of care in ALF; a strategy that has been associated with a decline in mortality from cerebral oedema. (Bernal et al., 2013) Whilst both strategies are neuroprotective they have additional benefits in reducing neutrophil activation in ALF, a setting where significant adaptation is unlikely to have had time to develop.

Ammonia-induced cell volume changes along with failure of p38-MAPK activation appear to be critical factors in the dysfunction of neutrophils in patients with acute and chronic liver disease. A more detailed understanding of how ammonia affects changes in neutrophil kinase pathways is required. p38-MAPK agonists offer exciting new targets to abrogate neutrophil dysfunction and susceptibility to infection in liver disease and warrants further investigation.

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Chapter 9 – Discussion and directions for future work

This thesis is the most comprehensive work to date characterising circulating neutrophil dysfunction in patients with acute and chronic liver failure. The data demonstrate the presence of neutrophil swelling, impaired phagocytosis and enhanced neutrophil spontaneous oxidative burst in patients with acute and chronic liver failure and contributes to increased susceptibility to infection. In patients admitted to ICU with ALF and SALF changes to all aspects of neutrophil function persist until spontaneous recovery, death or LT. Furthermore, the morphological studies show that circulating neutrophils isolated from patients with both ALF and cirrhosis display striking phenotypic changes along with changes in surface receptor expression and reduced chemotactic activity. Ammonia contributes to neutrophil swelling and further impairs neutrophil phagocytosis and augments SOB. Modulators of p38-MAPK can reverse this dysfunction suggesting that activation of this pathway can protect against the ammonia-induced neutrophil swelling and dysfunction.

The main criticisms of this body of work are the heterogeneity of the patient groups and the failure to achieve recruitment targets in some of the planned patient cohorts thus reducing the statistical power of the study. Continued recruitment of patients into some of the cohorts may strengthen the robustness of some of the findings and detect more subtle changes.

9.1 Neutrophil dysfunction in ALF

Circulating neutrophils in patients with ALF and SALF demonstrate a significant reduction in neutrophil surface expression of CD16 (FcγRIII) thereby reducing the ability of the neutrophil to bind to opsonised microbes akin to patients with sepsis and MODS. Pronounced impaired of phagocytic activity of opsonised *E. coli* was also observed in neutrophils isolated from patients with ALF and particularly SALF. These functional differences appear to impact on survival form ALF/SALF, particularly phagocytic dysfunction with patients who died or required LT showing a lower NPA at presentation compared to non-LT survivors.

Surface receptor data for circulating neutrophils in patients with ALF and SALF did not show the expected changes with a significant reduction in neutrophil surface expression of CD16 (FcγRIII) being the only difference detected. This data should be interpreted with caution as data for surface receptor expression was not available on all samples particularly for CD11b and CD62L thus reducing the power of the study. The

expected sepsis phenotype, of enhanced expression of CD11b, reduced CD62L and stable levels of CD16 was not see in the septic controls cohort suggesting either a small sample size or activation of neutrophils in the healthy control cohort. Overall, the study did under-recruit in the healthy control (n=11) and septic control (n=6) cohorts as we initially planned to recruit 24 to each cohort. Indeed, the sepsis controls did not demonstrate the expected surface receptor changes and were not as unwell as expected particularly when the organ failure scores in the sepsis cohort were compared to the ALF cohort suggesting that the cohorts are not as well matched in terms of organ failure possible explaining the lack of surface receptor changes in the sepsis cohort. Clearly ALF and sepsis are different and the reported variation in CD16 levels could be real so further studies should be performed to confirm these findings particularly with an expanded array of surface receptors. Interestingly, in other work we have shown that enhanced neutrophil CD64 expression is seen in ALF in the absence of infection suggesting sterile inflammation can induce CD64 expression; a phenomenon previously only reported in sepsis supporting the theory that sterile inflammation and hepatic-derived DAMPs can induce changes in circulating neutrophils with the caveat that subclinical infection cannot be excluded. (Abeles et al., 2012)

The observation of a reduction in NPA in ALF/SALF cohorts akin to that frequently observed in sepsis, (Brown et al., 2006) may explain why patients with ALF exhibit phenotypic features of septic shock with microvascular dysfunction, hemodynamic instability, coagulopathy, encephalopathy, MODS and high levels of circulating pro-inflammatory cytokines. Why the severity of NPA is less so in those presenting with ALF compared to SALF is less clear and perhaps the answer lies in the chronicity of onset of neutrophil impairment. A significant proportion of the ALF cohort were acetaminophen-induced ALF (40%) characterised by a rapid onset of liver failure and a significant CARS response may not have developed yet, whereas the more insidious time course of SALF (70% seronegative autoimmune disease) may lead to an enhanced CARS response and a more severe reduction in phagocytic ability seen although interestingly IL-10 levels were comparable. The TEM studies show features of neutrophil senescence in patients with ALF and this may be induced through oxidative stress, enhanced neutrophil life-span or a failure of stress granulopoiesis to replenish these cells. Senescent neutrophils can show reduced phagocytosis thus offering a further

explanation for the changes seen as well as the ammonia-induced changes. I=indeed, this may explain the time-dependent nature of the defect.

It is disappointing that haemodialysis does not appear to have an effect on neutrophil phagocytic dysfunction; however, our study was underpowered to detect this change. It has been shown that CVVH can reduce plasma ammonia levels with greater ammonia clearance achieved with higher filtration volumes. (Slack et al., 2014) So it may be too early to dismiss this approach particularly as this aspect of the study was observational. However, CVVH is commenced at an early stage in many patients with acute and chronic liver failure for management of hyperammonaemia, acid-base disturbance and AKI and so whilst it would offer mechanistic support it is unlikely to change management. Other therapies such as albumin dialysis and plasmapheresis may modulate neutrophil function to a greater extent than CVVH through removal of cytokines, DAMPs and PAMPs and comparative studies are mandated.

The heterogeneity of the two groups in terms of aetiology of liver disease does mean that we may not be comparing like with like particularly in respect to the speed of onset of liver failure and the varying severity of disease. This may affect neutrophil dysfunction and the severity of the SIRS, subsequent CARS response and the varying potential for recovery. The timing of sampling may make a difference; whilst patients were sampled within 48-hours of admission this was dependent on the timing of transfer to the ICU which was referring centre dependent and on the dynamics of presentation. In AALF this was probably more consistent as all patients presented with hyperacute ALF whereas a number of patients with viral liver disease were transferred and de-escalated from ICU within 48-hours with rapidly improving encephalopathy and subsequent spontaneous recovery.

One criticism of previous studies of neutrophil dysfunction was that the clinical relevance of the documented changes was unclear. However, one of the critical findings form the data is that NPA on admission appears to be a predictor of spontaneous survival compared to conventional organ failure scores such as SOFA and MELD which did not predict poor outcome in this study. Trying to understand the relationship between neutrophil phagocytic dysfunction and poor prognosis therefore seems critical. The incidence of ‘culture positive’ sepsis was low in this ALF/SALF cohort overall and indeed the observed deaths could not be directly attributed to infection, suggesting phagocytic dysfunction is either a reflection of general immune activation or a specific factor related to the severity of the liver injury. Peak plasma ammonia levels

demonstrated a robust correlation with poor phagocytic function in SALF and high circulating levels of IL-10 and IL-17. Ammonia was previously shown to impair NPA and induce spontaneous OB in healthy neutrophils exposed to supraphysiological concentrations of ammonia *ex vivo* and in rats fed an ammonia-rich diet. (Shawcross et al., 2008a) The peak arterial ammonia concentration did not however correlate with impaired NPA in the ALF cohort but might be attributed to the impact of ammonia reduction by CVVH prior to neutrophil sampling. Interestingly LT, with associated normalisation of plasma ammonia levels resulted in rapid improvement of neutrophil phagocytic function within 72 hours but not complete reversal, which could be the result of ischemia-reperfusion phenomena, ongoing production of pro-inflammatory cytokines/SIRS or sepsis.

Pro- and anti-inflammatory cytokine profiles might be expected to show a closer association with neutrophil OB than phagocytic activity although this was not generally the case. Higher plasma IL-10 and IL-17 concentrations correlated with impaired NPA and may suggest the development of a CARS in this condition (Antoniades et al., 2008) with involvement of T-regulatory cells (T-regs). (Lewis et al., 2012) However, the condition with the highest levels of pro-inflammatory cytokines, AALF demonstrated only modest neutrophil dysfunction. CD4+CD25+CD127-FOXP3+ T-regs directly inhibit neutrophil function, promoting apoptosis and death when exposed to lipopolysaccharide through TLR4 expressed on their surface which inhibits pro-inflammatory activities. (Lewkowicz et al., 2006) This is an important role in the direct control of innate immune responses. Upon activation, these T-reg can either induce themselves or CD4+CD25-FOXP3-T effector cells to differentiate into IL-17A producing cells, Th17, in the presence of TGF-beta and/or IL-6. (Xu et al., 2007) In contrast to the role of T-regs on neutrophils, one of the functions of Th17 is to recruit neutrophils into inflamed tissue further increasing the antimicrobial response *in vitro* and *in vivo*. (Annunziato et al., 2008, Kolls et al., 2008)

Possible mechanisms to explain the observed reduction in NPA could include direct toxicity to neutrophils through cell swelling, neutrophil senescence mediated through oxidative stress, a CARS response or suppression by T-regs. The mechanisms seem complex and may be multiple but understanding the mechanisms seems central to unlocking the immune pathology and realising the potential to improve outcome in this condition. Interestingly, NPA did not show any association with SIRS score, organ failure scores or pro-inflammatory cytokine levels. The SIRS score has poor sensitivity

and narrow range with many parameters such as tachycardia, tachypnoea, respiratory alkalosis, fever and neutropenia being features of ALF and are therefore unlikely to be useful in this setting. Organ failure scores are relatively blunt tools to correlate with NPA and indeed, would be expected to correlate better with SOB. In addition the heterogeneity of the cohort here probably limits my findings. In particular I would not exclude an effect of pro-inflammatory cytokines on the basis of this study alone. Measurement of pro-inflammatory cytokine levels in the serum has limitations with a variation in concentrations away from the liver and so the effects of priming with cells circulating through the liver bed and local concentrations or gradients may be of greater importance. Indeed, cytokine effects can be additive and peripheral levels may be reduced by CVVH. Cytokines mediate indirect effects on neutrophils through the activationof the vascular endothelium or other inflammatory cells that modulate function. Certainly an expanded range of cytokines, DAMPs and PAMPs need to be considered in future studies and multivariate analysis should be employed to exclude confounding factors.

There was no evidence of enhanced neutrophil spontaneous production of ROS contributing to the development of MODS and poor outcomes in ALF/SALF in this study. Circulating neutrophils may not reflect activation of marginated neutrophils in the liver and other vascular beds within organs such as the lung and kidney so it is difficult to draw firm conclusions. In addition, ALF and SALF patients are a heterogeneous patient group who are prone to deteriorating rapidly necessitating a number of invasive interventions such as haemofiltration, mechanical ventilation and mild hypothermia potentially influencing neutrophil function and which are difficult to control for constituting the main weakness of this study. Furthermore, the empirical use of potent broad-spectrum antibiotics and anti-fungals as standard of care in this study is also likely to have abrogated any increased susceptibility to developing sepsis in this cohort.

9.2 Neutrophil dysfunction in cirrhosis

In patients with stable cirrhosis, my data confirm that primed dysfunctional neutrophils circulate with impaired chemotaxis and phagocytic function. The circulating neutrophil dysfunction predisposes to infection and predicted the development of infection, organ dysfunction and survival at 90-days and 1-year. Furthermore, patients who displayed evidence of increased oxidative stress with spontaneous production of ROS from circulating neutrophils were susceptible to the development of HE and

AoCLFwith associated poor outcome. This supports a central role for circulating dysfunctional neutrophils in the pathogenesis of extra-hepatic organ dysfunction in cirrhosis.

Secondly, TEM studies confirmed that ammonia-induced neutrophil swelling correlated with neutrophil phagocytic dysfunction, swelling and activation of p38-MAPK. Thirdly, mechanistic studies demonstrated that abrogation of the neutrophil dysfunction seen in liver failure can be achieved by pre-incubation of *ex vivo* neutrophils with specific modulators of p38-MAPK. Neutrophils exposed to the p38-MAPK agonist isoprotorenol recovered phagocytic capability offering a novel therapeutic target to prevent that may reduce the development of HE and AoCLF in cirrhosis.

Morphological studies using TEM have thrown up some very interesting observations by confirming that patients with cirrhosis have an increased neutrophil cross sectional area compared to controls, correlating with increasing plasma ammonia levels. Furthermore, TEM studies have revealed profound abnormalities in the neutrophil ultrastructure with loss of rounded shape, extensive vacuolisation, pale cytoplasm and paucity of mitochondria. In the setting of liver disease these findings are novel and may signify neutrophil senescence either as a consequence of neutrophil oxidative stress from enhanced spontaneous ROS production via NADPH oxidase, or a prolonged neutrophil life-span as a consequence of reduced clearance through apoptosis.

Neutrophil senescence has previously been described and renders neutrophils unable to respond to chemoattractants, carry out respiratory burst or degranulate. (Whyte et al., 1993) Interestingly, the chemotaxis experiment shows a variable response to the chemoattractant fMLP, with neutrophils from patients with cirrhosis showing an activated phenotype with loss of CD62L. However, the ability of neutrophils to migrate following fMLP stimulation appeared to be impaired although findings were to some extent heterogenous. The findings of the chemotaxis assay would therefore support neutrophil senescence. No apparent association with plasma arterial ammonia concentration was seen. This supports earlier findings by Fiuza et al. who showed an impaired ability of neutrophils in patients with cirrhosis to migrate using a skin window technique. (Fiuza et al., 2000) Interestingly ammonia has been reported to inhibit the fMLP receptor but although the chemotaxis response is impaired the activation response is not, so ammonia may not be the aetiological agent here. Circulating neutrophils in patients with cirrhosis retained the ability to generate an ESOB and degranulation appeared to be increased with the increased surface expression of CD11b although the response to other chemo-

attractants was not specifically investigated. TNFα levels are increased which along with GM-CSF can extend neutrophil life-span as well as LPS priming both of which could be potential mechanisms for the altered chemotaxis response.

Clearly data on apoptosis is needed and it is interesting to note that G-CSF has been shown to enhance survival in patients with AoCLF who were treated with intermittent dosing over a month. These patients had fewer episodes of sepsis and improved outcome at 2-months associated with increased circulating numbers of neutrophils and CD34+ cells (Garg et al., 2012) suggesting that G-CSF enhances mobilisation of new neutrophils from the bone marrow.

The TEM studies, need to be reproduced particularly with a less intensive method perhaps using techniques such as oil immersion light microscopy or confocal microscopy to make it more practical to study in greater detail. Interestingly, using the GMFI of the forward scatter as a marker of cell volume did not intimate any difference between control and disease neutrophils, which is probably a consequence of sample preparation. Whole blood preparation for assessment of neutrophil cell volume involved a density graded centrifugation technique with Polymorphoprep™, which involves a prolonged exposure to a hyperosmolar solution with subsequent restoration of osmolality, after separation, by addition of 0.45% saline solution. Normal cells may be more resistant to the effects of the hyperosmolar solution on cell volume than cells from patients with liver disease. Ideally a whole blood technique would prevent this issue as well as reducing handling and maintaininga constant temperature.

The data show that circulating neutrophils in cirrhosis exhibit a classical activated phenotype of cell surface receptors, with enhanced expression of CD11b, loss of CD62L and unchanged levels of CD16. In addition neutrophils from patients with cirrhosis show a reduced capacity to phagocytose microbes with impairment of chemotactic migration following fMLP stimulation. The caveat in interpreting the up-regulation of neutrophil surface receptor expression in liver disease, particularly CD62L, is that there is a high degree of CD62L in the maturating pool within the BM and these can be released during stress granulopoiesis from the BM. L-selectin is lost through neutrophil aging and following corticosteroid exposure. (van Eeden et al., 1999) Upregulation of the β2-integrins CD18 and CD11b is considered a sensitive marker of neutrophil activation and a whole blood preparation is considered a preferable assay to prevent activation particularly following the cell lysis and wash steps and I would look to develop this assay in the

future. There are further markers of activation, particularly the β1-integrin CD49d (VLA4), the high affinity Fcγ receptor CD64, the CXCR1 chemokine receptor and the PRR TREM-1 and these could be assessed by using multiple antibodies with different fluorochromes on the same assay. Other concerns result from the heterogeneity of the group and exclusion of infection in the recruited cohort. A number of the patients in the stable cirrhosis cohort had been clear from infection for only 7-days and questions have to be raised as to whether this was long enough and had infection really been excluded at recruitment and subsequent sampling.

The finding of an enhanced spontaneous production of ROS from circulating neutrophils in patients who go on to develop infection and organ dysfunction or HE may hold the key to understanding why some patients with cirrhosis are susceptible to developing MODS and HE. Indeed, patients with cirrhosis who have increased basal production of ROS appear to be at the highest risk for developing complications and MODS. This is likely to be driven by the systemic pro-inflammatory milieu that bathes these neutrophils with elevated levels of TNFα, IL-6, IL-8 and IL-10 generated principally as a consequence of bacterial infection, with complicating infection being seen in 23% of patients with HE and 58% of patients with AoCLF. Enhanced bacterial translocation of PAMPs across a leaky gut (Neugebauer et al., 2008) or from 'sterile' hepatic inflammation (DAMPs such as HMGB-1) may explain other cases. These findings support the hypothesis that bacterial driven (PAMPs) or 'sterile' hepatic inflammation induces a dysfunctional response in circulating neutrophils and through homing to other endothelial beds can induce oxidative stress, organ-injury and the development of the clinical syndromes of AoCLF and HE. Similar findings have been shown in patients with acute alcoholic hepatitis where an increase in SOB is observed which correlates with severity of liver disease. (Parlesak et al., 2003, Mookerjee et al., 2007b) Interestingly, the phenomenon of increased SOB appears to be independent of plasma ammonia levels and indeed the mechanisms which lead to neutrophil NPA being reduced seems to be separate from that of enhanced resting OB.

The finding however, that elevated serum transaminases and hyperbilirubinaemia are associated with higher plasma levels of IL-17 and a lower phagocytic capability in AoCLF supports hepatic inflammation as being an important driver of neutrophil phagocytic dysfunction. IL-17 is a critical mediator of neutrophil recruitment. IL-17 secretion from effector $CD4^+$ T-cells is thought to be an important mediator of innate

immune function and the development of chronic inflammation, (Qian et al., 2010) and IL-17 secreting hepatic cell infiltrates have been shown to be present in patients with acute alcoholic hepatitis. (Lemmers et al., 2009)

Ammonia-induced neutrophil swelling correlates with neutrophil phagocytic dysfunction and activation of the p38-MAPK pathway. Neutrophil swelling correlated with peak arterial concentration and could be abrogated *ex-vivo* following incubation with the p38-MAPK agonist isoproterenol. Further support of a central-role for activated p38-MAPK in NPA comes from the observation that in patients with diuretic intolerant/resistant ascites the concomitant use of propranolol was associated with worsening of NPA. This may offer an explanation as to why Serste and colleagues recently identified increased mortality in patients with refractory ascites treated with propranolol. (Serste et al., 2010) Propranolol increases neutrophil motility by promoting increased intracellular cyclic GMP activity (Anderson and van Rensburg, 1979) and thus might be postulated to have a beneficial effect in cirrhosis. Propranolol inhibits p38-MAPK phosphorylation and can therefore suppress neutrophil phagocytic function. (Busse and Sosman, 1984) Reduced NPA in concert with increased bacterial DNA translocation from the gut into the portal vein in patients with refractory ascites might thereby culminate in hepatic endothelial dysfunction, systemic endotoxemia, and resultant systemic inflammatory response. (Bellot et al., 2010)

The observed association between elevated plasma ammonia concentration and impaired NPA replicates Dr Shawcross and colleagues previous observations. (Shawcross et al., 2008a) Acute increases in plasma arterial ammonia concentration induced by a simulated upper gastrointestinal bleed have been shown to induce neutrophil swelling and phagocytic dysfunction in patients with stable cirrhosis. The adverse impact of ammonia exposure on neutrophil function can be abrogated *ex vivo* following incubation of the neutrophils with an agonist of the p38-MAPK pathway. (Shawcross et al., 2008a) It has been postulated that ammonia-induced neutrophil swelling induces phosphorylation of p38-MAPK which serves as an important cell osmoregulator. (vom Dahl et al., 2001) p38-MAPK is also upregulated during neutrophil priming and activation, with down-stream effects including the promotion of apoptosis and the activation of pro-inflammatory transcription factors. However, the association between ammonia and neutrophil phagocytosis remains circumstantial and p38-MAPK activation may occur through other mechanisms. Indeed the correlation between NPA in patients with cirrhosis and serum ammonia is modest at best and although it has been reproduced other factors

such as neutrophil count and gammaglobulin levels show a similar magnitude of effect. Multivariate analysis of potential serological factors is required in an expanded cohort or within a single aetiology cohort in order to reduce confounders. The correlation with neutrophil count is interesting and a possible explanation may be that patients with cirrhosis who can mount a neutrophilia may be able to release younger more vigorous neutrophils from the bone marrow that can improve NPA. Clearly loss of opsonins such as IgG will reduce NPA as has previously been shown with low complement levels in ascites. (Such et al., 1988) The finding of ammonia levels correlating with NPA is a consistent finding, however, the exact mechanism through which ammonia causes neutrophil swelling is yet to be elucidated. Interestingly, ex-vivo neutrophils incubated with ammonia appeared to inhibit PMA-activated oxidative burst more profoundly that ESOB. PMA is known to activate p38-MAPK following activation of PKC and subsequent phosphorylation and ammonia's effects therefore appears to be specific to this pathway whereas *E. coli* can still generate a burst via alternative pathways. This then begs the question that if cell swelling is implicated in neutrophil malfunction, is this achieved through similar mechanisms to astrocytes by the intracellular conversion of glutamate to glutamine or other toxic effects on mitochondria?

The role of p38-MAPK appears to be fascinating and it seems this multifunctional kinase is able to protect against the cell swelling effects of ammonia. The more important question is perhaps what causes inactivation of phosphorylated p38-MAPK? Is it reduced phosphorylation perhaps due to oxidative stress or enhanced breakdown? What is clear is that there is reversibility in the process and recovery can be seen with isoproterenol which promotes phosphorylation of p38-MAPK. Isoproterenol activation of p38-MAPK appears to be acting through an alternative mechanism via cAMP activation. Interestingly, β_2-adrenoceptor agonists such as adrenaline and noradrenaline lead to reduction in the bacteriocidal activity of the neutrophil against *S. aureus*, *E. cloacae* and *Proteus ruttgeri* without decreasing phagocytosis of bacteria. (Qualliotine et al., 1972) Although both are less potent than isoproterenol and are often present in elevated levels in patients with cirrhosis and ALF/SALF, the relevance of these effects *in vitro* is unclear. It is a shame that the CBA assay did not yield any useful results as this would have shed some light on the level of background phosphorylated p38-MAPK activation.

Perhaps the most powerful observation of this study is that baseline circulating neutrophil dysfunction is a robust predictor of outcome and 90-day and 1-year survival.

Baseline resting neutrophil OB of ≥12% predicted 90-day survival with an AUROC of 0.81, with survivors at 90-days having a significantly lower resting OB. Impaired NPC is also an important determinant of 90-day mortality with an AUROC of 0.83 with survivors at 90-days and 1-year having a significantly higher NPC. This implies that neutrophil biomarkers can predict medium-term (90-day) and longer-term (1-year) outcome in patients with stable cirrhosis. This also supports the findings of Mookerjee and colleagues who showed neutrophil dysfunction to be an important outcome predictor in acute alcoholic hepatitis. (Mookerjee et al., 2007a)

Overall the results allow us to speculate that circulating neutrophil dysfunction in liver disease appears to be a maladaptive response to chronic inflammation with neutrophils 'dressed for a party but with nowhere to go' being maintained in a constantly primed state within the circulation which eventually culminates in cell exhaustion leading to senescence. There are associations with markers of hepatic inflammation, pro- and anti-inflammatory cytokines as well as plasma ammonia levels. The implication seems to be that in chronic liver disease despite raised levels of anti-inflammatory cytokines such as IL-10 there is incomplete resolution of the inflammatory response and perpetuation of low grade SIRS through LPS priming via TLRs. Reduced CD62L expression may indicate a reduced ability to marginate to the vascular endothelium but as yet the activation state of other adhesion molecules in unknown. We can speculate that the persisting primed state combined with ammonia-induced cellular swelling and reduced clearance through apoptosis leads to neutrophil senescence and reduction in functions such as phagocytosis and chemotaxis. One difficulty with this theory is the reversibility observed with p38-MAPK modulators and indeed the data still leaves many questions unanswered. Further work needs to be carried out to acquire supporting evidence for which of these mechanisms assumes most importance in the functional changes observed.

9.3 Conclusion

Overall we can see that the neutrophil dysfunction both in ALF and cirrhosis is myriad and complex. These findings support the hypothesis that a combination of bacterial translocation, 'sterile' hepatic inflammation and ammonia induce a dysfunctional response in circulating neutrophils. Through homing to other endothelial beds these cells induce oxidative stress and organ-injury. In addition, other mechanisms

of neutrophil dysfunction including senescence resulting from the ravages of oxidative stress and defective apoptosis have been suggested. Agonists of p38-MAPK can improve phagocytic ability and suppress resting neutrophil ROS production offering a novel therapeutic target to prevent complicating HE and sepsis in cirrhosis.

Chapter 10 - Directions for Future work.

My studies have raised more questions than answers and in particular to achieve the aims of improved mechanistic understanding and the eventual development of neutrophil modulating therapies to improve outcomes in liver failure, further work needs to be done. Indeed, I feel I have only just scratched the surface and a much larger body of work needs to be done to understand the neutrophil changes which will not only have implications for liver failure but also for other conditions such as severe sepsis.

A comment needs to be made on the rigor of the study and patient selection for future studies. As discussed above, one of the key problems which makes interpretation difficult in this observational study is the open inclusion and exclusion criteria meaning that there are many factors not controlled for such as age, varying immune activation, medication and co-existent conditions. Tighter inclusion and exclusion criteria are required to avoid heterogeneity between groups and patients need to be screened rigorously for sepsis prior to inclusion. In particular concentrating on a single aetiology of liver disease such as ARLD may avoid subtle differences in the immunopathology that may be present in other aetiologies of chronic liver disease. Sub-groups should include patients on and off prophylactic antibiotics and non-selective β-blockers. I would also point out that it is important that the control group should exclude smokers and particularly those who have done vigorous exercise in the last 24-hours as I have found that these factors can affect neutrophil function markedly.

The neutrophil phagocytic dysfunction observed in cirrhosis and ALF/SALF is profoundly interesting as it appears central to the immunodeficiency observed and triggers a cycle of infection, enhanced neutrophil burst and SIRS, ROS induced organ-failure and if the patient survives a prolonged CARS phase predisposing to further cycles of infection and ultimately in many patients death. Better understanding of the pathways through which this is mediated must include studies into the cytoskeletal rearrangements that take place within the dysfunctional neutrophil particularly changes to the Ca^{2+}-dependent mobilisation and F-actin assembly. These studies should be complemented by observations of neutrophil granule numbers, membrane association and release resulting in receptor clustering around the pseudopodium and phagosome cup through imaging and flow cytometry studies.

Neutrophil phagocytic dysfunction is a consistent finding across patients with both ALF and cirrhosis but severity is variable and is incompletely explained by liver disease severity. Moreover, the SALF work suggests it worsens in the presence of

persisting hepatic inflammation. If the neutrophil defects are akin to sepsis then focusing on mechanisms such as priming, senescence, inflammatory cytokines, neutrophil band-forms and suppression from T-regs should be the next step. In liver disease this would be best explained by low-grade bacterial translocation. A question remains as to the role of sterile inflammation with systemic release of DAMPs and whether a similar sepsis phenotype can be generated in the absence of infection. The aspect of liver disease that set it aside from sepsis includes hyperammonaemia and there is much evidence to support its pathogenic role. Defining the neutrophil phenotype seems critical to show the similarities and subtle nuances between sepsis and acute and chronic liver failure in order to guide and focus mechanistic studies. Interestingly, phagocytic dysfunction does not appear to be worse in cirrhosis with superadded infection such as AoCLF. Indeed our findings show that neutrophils in patients with sepsis show a less severe phagocytic dysfunction, going against the perceived wisdom but due to the small numbers of septic controls and low organ failure score this should be viewed with some scepticism. Given the redundancy in neutrophil signalling it seems plausible however that more than one mechanism may be involved in suppression of neutrophil phagocytosis so there may still be a role for pro- and anti-inflammatory cytokines and priming agents and so they should not be discounted at this point. If we assume that the p38-MAPK pathway is critical, one possible unifying mechanism may be that DAMPs and PAMPs through pathways such as TLR activation, supress p38-MAPK activation thus reducing the neutrophils ability to withstand the toxic effects of ammonia leading to swelling and phagocytic dysfunction. Microbial infection tips the balance in these primed circulating neutrophils probably through changes in the SIRS/CARS axis, likely independent of ammonia, to produce toxic neutrophils with enhanced SOB and possibly through granule release or NET formation can lead to damage of the vascular endothelium and organ dysfunction.

Portal hypertension and porto-systemic shunting is clearly important as a source of endotoxin which leads to neutrophil priming through TLR-4 and ammonia. The role of DAMP's such as HMGB-1 and other agents which are known to affect the p38-MAPK pathway need to be investigated. Initially I would suggest an expanded cohort of patients should be recruited with a single aetiology and multivariate analysis should be performed on possible aetiological agents. This may be difficult knowing what to test for as well as methodological difficulties such as the notorious difficulty in measuring endotoxin levels. Mass spectrometry methods such as matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) could be used to identify molecular signatures which correlate with

phagocytic dysfunction. If samples were collected in an identical fashion they could be combined with this data. Particular attention should be paid to studying an appropriate number of healthy controls and septic controls who are more closely matched in terms of organ failure.

Pharmacological experiments using plasma from patients with liver failure on healthy neutrophils with the addition of inhibitors such as anti-TNF drugs (e.g. infliximab), TLR-2 and TLR-4 receptor blockers and β-adrenoceptor agonists and antagonists (e.g. isoproterenol, noradrenaline and propanolol) will subsequently be helpful.

Ammonia is clearly important but again associations are variable and there are clearly other factors involved which may increase or reduce the susceptibility to ammonia. It may be that factors such as neutrophil senescence or uncharacterised serum humoral factors that inhibit the phosphorylation of p38 MAPK may increase the susceptibility of neutrophils to the effects of ammonia. In particular the effects of β-blockade on neutrophil dysfunction merits further study to understand if the observed effect of these drugs on neutrophils is robust and to confirm if this is affected through the p38-MAPK pathway. This would have important implications regarding the use of these drugs in the prophylaxis of variceal haemorrhage in advanced cirrhosis. Pre-incubation of normal neutrophils with serum from patients with liver disease followed by ammonia exposure should be done. Specifically, looking for protective factors against ammonia exposure such as sera from healthy controls, albumin, gamma globulin or G-CSF. If G-CSF were beneficial then clinical studies looking at the effect of G-CSF in patients with sepsis or AoCLF would be the next logical step with examination of neutrophil functional indices as well as episodes of infection and survival as measurable outcomes.

One of the important and controversial hypotheses is that neutrophils are involved in the development of tissue damage and organ dysfunction in patients with liver disease and more evidence needs to be developed in this regard. There are 3 areas to focus on, firstly looking at an expanded set of surface expression of adhesion molecules such as selectins and integrins, secondly examining margination within the vasculature of these organs using flow-cell experiments looking at adhesion *in-vitro*, and thirdly histological evaluation of tissue such as liver explants from patients who undergo transplantation. Tissue samples from other organs are difficult to obtain as few patients undergo renal or brain biopsies and it would be unethical to do so unless at post-mortem. Animal models

such as the bile-duct ligated rat could be used but there are important immunological differences which need to be born is mind with fewer circulating neutrophils being present. More powerful experiments would be looking at before and after following modulation of neutrophil function either following treatment with isoproterenol or techniques such as neutrophil removal utilising columns. The role of other neutrophil functions such as protease release and NET formation should be included, as a modest increase in ROS production may not be enough on its own to cause organ dysfunction.

Another question is to define the phenotype and functionality of neutrophils which have migrated across the vascular endothelium as these cells are at the coal face dealing with invading organisms and they may not show the functional defects that are characteristic of the circulating neutrophil population. Experiments on neutrophils that have trans-migrated across the vascular epithelium in humans have traditionally used a skin window technique which artificially creates an often painful blister and the applicability of neutrophil changes observed by this technique is controversial. Ascitic fluid is another potential source of neutrophils that have crossed the vascular endothelium particularly in patients with decompensated cirrhosis. Ascitic fluid is often removed as a diagnostic procedure to confirm SBP and also large volumes are removed as a palliative procedure in patients with tense ascites. This would potentially provide a source of migrated neutrophils to compare although methods to optimise yield would need to be developed. Experiments to isolate and look at the phenotype and functionality of migrated neutrophils from patients with SBP needs to be done. Some authorities now recognise that reverse migration can occur back into the circulating pool and defining neutrophil sub-sets may allow a better understanding.

Examination of circulating neutrophils within portal blood would be valuable if enhanced priming due to increased bacterial translocation of products such as endotoxin and LTA occurs. Portal blood from patients with cirrhosis or ALF/SALF can be obtained either directly at time of LT or during TIPS procedures either for variceal bleeding or intractable ascites. The assumption is that neutrophil priming occurs in this circulation from factors released in the liver or from porto-systemic shunting and study of the inflammatory milieu in this circulation is more likely to identify causative agents. Again a number of bacterial molecular signals have been defined by MALDI-TOF and examination of the plasma by this technique is likely to yield information about the degree of bacterial translocation and PAMP's present. Comparing peripheral circulating neutrophils at the same time as similarities and differences could be sought.

TEM should be reserved for looking at the ultrastuctural changes present in neutrophils from patients with liver failure in an expanded cohort to ensure that these changes are reproducible and how they compare to patients with sepsis. The hypothesis that neutrophil senescence may in part explain neutrophil dysfunction needs to be further explored. In addition looking for mitochondrial changes that are typical of oxidative damage should be sought and careful assessment of phagosome structure in response to *E. coli* exposure can be looked at following immunogold labelling of F-actin to define defects. Dynamic confocal microscopic studies of neutrophil behaviour would complement these studies. (Allen, 2007) Granule structure and contents can be studied using immunogold labelling of MPO or the cytosolic NADPH subunits ($gp91^{phox}$ or $p22^{phox}$) to try to better understand the state of NADPH activation and efficiency of neutrophil killing as well as depletion.

As already explained above, neutrophils display an activated phenotype of surface receptor expression with upregulation of CD11b, reduced CD62L and unchanged CD16 levels as typified in sepsis. Other receptors which could be looked at include the integrins: CD11b/CD18; and CD49d (VLA4), PRRs: TLR-2; TLR-4; and Trem-1, Fcγ receptors: CD32, CD64 and chemokine receptors CXCR2 and CXCR4 as per Table 2-2. This would allow determination of the level of neutrophil activation and comparison to the sepsis phenotype. Upregulation of the β2-integrins CD18 and CD11b is considered a sensitive marker of neutrophil activation and a whole blood preparation is considered a preferable assay to prevent activation particularly following the cell lysis and wash steps and I would look to develop this assay in the future. CD18 upregulation originates from specific granules with CD11b being present in secretory granules along with the complement receptor CD35. CD18/CD11b as well as representing neutrophil activation serves as a marker of neutrophil degranulation. Neutrophil subsets have recently been defined with the observation that reverse trans-endothelial migration can occur from the adluminal-to-luminal space. (Beyrau et al., 2012) In addition CD32 can be used as a marker to exclude eosinophils although the numbers present in patients with liver disease will be small.

Further studies of neutrophil chemotaxis should be performed on neutrophils isolated from more selective cohorts of single aetiology liver disease at different stages of presentation and following infection to ensure that the findings are reproducible. Looking at neutrophil migration responses to other chemokines particularly liver chemokines, such

as osteopontin, may be more relevant to the pathology encountered to ensure that the defects remain.

Neutrophils do not operate in isolation and the immunopathology affects other innate and adaptive immune cells with PBMC's and IL-17 T-cells being of particular interest and co-culture studies should be performed. Relevant to this is the role of neutrophils in orchestrating the inflammatory response through the production of cytokines and initial intracellular cytokine studies on T-cells co-cultured with neutrophils show promise. These experiments are important as it is increasingly recognised that neutrophils contribute themselves to the inflammatory milieu recruiting monocytes, dendritic cells, NK cells and T-cells as well as promoting survival and differentiation of B-cells,

A broader understanding of the neutrophil dysfunction should be sought looking at chemotaxis, NADPH oxidase dysfunction, cytoskeletal re-arrangements and neutrophil granules as this may help elucidate a common pathway for the development of dysfunction. Further mechanistic studies are critical particularly as blanket therapies such as corticosteroids are a double-edged sword. Looking at intracellular signalling right form the level of TLR and FcγR receptors down to p38-MAPK and other intracellular kinase cascades through to the generation intracellular cytokines is warranted. The availability of small molecule inhibitors or murine knockout models may ultimately offer translational targets and therapies. Currently available therapies such as plasmapheresis in ALF and G-CSF in AoCLF could offer hope in the meantime

There is clearly a greater needfor therapeutic trials to look at potential *in vivo* modulators of neutrophils. G-CSF holds promise, being able to promote release of neutrophils from the bone marrow and reduce apoptosis, and has shown some promise in patients with ALF and AoCLF in terms of reducing mortality and septic episodes respectively. The effect on neutrophil function was not studied and this would provide a useful insight. There is controversy as to what the effect of a higher proportion of immature band-forms would have and delaying apoptosis in older cells may be undesirable. Studies with *ex-vivo* neutrophils could be up-scaled to human clinicial trials if shown to be beneficial as studying *ex vivo* neutrophils in isolation will only give us a limited insight. The effect of immunosuppressant drugs on neutrophils needs to be better understood in particular to look at the effects in patients treated with corticosteroids especially using sequential samples with patients as their own controls and controlling

tightly for sepsis. The effect of intravenous immunoglobulin, plasmapheresis and CVVH should be studied either *ex vivo* or *in vivo*. Other potential targets include IL-33 a member of the IL-1 superfamily and TLR4-activated platelets. IL-33 appears to be a key regulator of neutrophil function during SIRS. In mouse models administration of IL-33 prevented the down-regulation of CXCR2 on circulating neutrophils and thus increased migration to inflamed tissues and promoted bacterial clearance. (Alves-Filho et al., 2010) In addition, TLR4-activated platelets have been shown to bind adherent neutrophils during sepsis and promote NET formation, which may lead to bacterial trapping but also to endothelial and tissue damage *in vivo* and *in vitro.* (Clark et al., 2007)

Overall, neutrophil dysfunction underpins much of the immune dysfunction present in acute and chronic liver failure. The causes appear complex but ammonia plays a central role. Specific targets and therapeutic manipulation holds promise for the future in reducing the incidence of sepsis and development of MODS.

Chapter 11 – Publications and Abstracts presented at National and International Conferences

11.1 Publications

1) Ammonia and the neutrophil in the pathogenesis of hepatic encephalopathy in cirrhosis: Shawcross DL, Shabbir SS, **Taylor NJ**, Hughes RD. Hepatology. 2010 Mar;51(3):1062-1069. Review.
2) The impact of organ dysfunction in cirrhosis: survival at a cost? Shawcross DL, Austin MJ, Abeles RD, McPhail MJ, Yeoman AD, **Taylor NJ**, Portal AJ, Jamil K, Auzinger G, Sizer E, Bernal W, Wendon JA. J Hepatol 2012 May;56(5):1054-62. Epub 2012 Jan 13.
3) Circulating Neutrophil dysfunction in acute liver failure. Taylor NJ, Nishtala A,Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, Wendon JA, Shawcross DL. Hepatology 2013 Mar;57(3):1142-52. Epub ahead of print 2012 Dec 12.
4) Neutrophil CD64 expression is elevated in acetaminophen-induced acute liver failure. Abeles RD, **Taylor NJ**, Vijay GK, Ryan J, Tranah TH, Bernal W, Wendon JA, Shawcross DL. Am J Respir Crit Care Med. 2012 Nov 15;186(10):1058-9.
5) The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. **Taylor NJ**, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, Wendon JA, Shawcross DL. Alimentary Pharmacology and Therapeutics, 2014 Sept;40(6):705-15. Epub 2014 Jun 25.
6) Editorial: neutrophil dysfunction in patients with cirrhosis–authors reply. **Taylor NJ** and Shawcross DL. Alimentary Pharmacology and Therapeutics, 2014 Oct;40(8):987.

11.2 Abstracts

British Association for the Study of the Liver

1) **NJ Taylor**, A Nishtala, F Lin, RD Abeles, J O'Grady, J Wendon, Y Ma, D Shawcross. Neutrophil dysfunction: The missing link between ammonia, infection and hepatic encephalopathy? Gut 2010; 59 (Suppl 2):A22

2) **NJ Taylor**, A Nishtala, F Lin, RD Abeles, W Bernal, J Wendon, Y Ma, D Shawcross. Neutrophil dysfunction: A potential biomarker of poor prognosis in acute liver failure? Gut 2010; 59 (Suppl 2):A31.
3) D Shawcross. A Nishtala, **NJ Taylor**, JA Wendon. Ex vivo treatment of neutrophils with a P38-MAPK agonist in patients with liver failure improves their bactericidal capacity. Gut 2010; 59 (Suppl 2):A27.
4) **Taylor NJ**, Ma Y, Abeles RD, Hussein M, Auzinger G, Vergani D, Bernal W, Wendon JA, Shawcross DL. Neutrophil-induced oxidative stress and low plasma IL-10, and not ammonia, are associated with the development of hepatic encephalopathy in patients with end-stage cirrhosis (Abstract) BASL 2009.
5) **Taylor NJ**, Ma Y, Abeles RD, Hussein M, Auzinger G, Vergani D, Bernal W, Wendon JA, Shawcross DL. Cirrhosis leads to impaired neutrophil phagocytosis and spontaneous oxidative burst which correlates with increasing IL-10 concentration and is reversed following liver transplantation (Abstract) BASL 2009.

British Society of Gastroenterology

1) **NJ Taylor**, RD Abeles, Y Ma, JA Wendon, DL Shawcross. A compensatory anti-inflammatory response syndrome (CARS) triggered by neutrophil-induced oxidative stress is associated with low grade hepatic encephalopathy in patients with advanced cirrhosis. Gut 2010;
2) **NJ Taylor**, RD Abeles, M Hussein, Y Ma, JA Wendon, DL Shawcross. Impaired neutrophil phagocytic capacity in patients with advanced cirrhosis is related to the development of ammonia-induced neutrophil swelling. Gut 2010;

European Association for the Study of the Liver

1) **NJ Taylor**, RD Abeles, Y Ma, JA Wendon, DL Shawcross. A compensatory anti-inflammatory response syndrome (CARS) triggered by neutrophil-induced oxidative stress is associated with low-grade hepatic encephalopathy in patients with advanced cirrhosis. J Hepatol 2010; 52 (Suppl 1):S216
2) **NJ Taylor**, RD Abeles, M Hussein, Y Ma, JA Wendon, DL Shawcross. Impaired neutrophil phagocytic capacity in patients with advanced cirrhosis is related to the

development of ammonia-induced neutrophil swelling. J Hepatol 2010; 52 (Suppl 1):S216

American Association for the Study of Liver Diseases

1) **Taylor NJ**, Ma Y, Abeles RD, Hussein M, Auzinger G, Vergani D, Bernal W, Wendon JA, Shawcross DL. Cirrhosis leads to impaired neutrophil phagocytosis and spontaneous oxidative burst which correlates with increasing IL-10 concentration and is reversed following liver transplantation. Hepatology 2009; 50 (S4): 121A (#308)
2) **Taylor NJ**, Ma Y, Abeles RD, Hussein M, Auzinger G, Vergani D, Bernal W, Wendon JA, Shawcross DL. Neutrophil dysfunction in acute liver failure defines those patients who have a poor prognosis and is reversed following liver transplantation. Hepatology 2009; 50 (S4): 115A (#249)

International Society for Hepatic Encephalopathy and Nitrogen Metabolism

1) D Shawcross. A Nishtala, **NJ Taylor**, JA Wendon. Ex vivo treatment of neutrophils from patients with acute and acute-on-chronic liver failure and hepatic encephalopathy with isoproterenol improves neutrophil phagocytosis (Abstract 2010)

International Symposium on Hepatic Encephalopathy

1) **Taylor NJ**, Ma Y, Abeles RD, Hussein M, Auzinger G, Vergani D, Bernal W, Wendon JA, Shawcross DL. Neutrophil-induced oxidative stress and low plasma IL-10, and not ammonia, are associated with the development of hepatic encephalopathy in patients with end-stage cirrhosis (Abstract) 2009.

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Appendices